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Management Approaches for Ceratocystis Wilt and Canker Disease in Acacia Plantations

by

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Declaration of Originality

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Statement of Co-Authorship

My doctoral studies were carried out during my enrolment in PhD degree in the Tasmanian Institute of Agriculture, University of Tasmania between 2015 and 2018. The literature review and results described in chapters 2, 3, 4 and 5 have been prepared for submission to journals.

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List of Abbreviations

BCA	Biological Control Agent
CFU	Colony Forming Unit
CTAB	Cetyl Trimethyl Ammonium Bromide
DAI	Day after Inoculation
DI	Discolouration Index
EB	Endophytic Bacteria
ISR	Induce Systemic Resistance
LAI	Leaf Area Index
MAI	Mean Annual Increment
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
SAR	Systemic Acquired Resistance
USDA ARS	United States Department of Agriculture – Agricultural Research Service
WAI	Week after Inoculation

Abstract

Acacia species are exotic hardwood trees extensively planted in SE Asia. Due to their rapid growth, good wood quality and tolerance to a wide spectrum of environmental conditions, they are important economically, not only for timber products, but also as raw material for significant pulp and paper industries in Indonesia.

A devastating disease of plantation *Acacia mangium* in SE Asia is caused by a fungal pathogen belonging to the genus *Ceratocystis*. This fungal pathogen, *Ceratocystis manginecans*, causes stem cankers and rapid wilting of trees. At the start of my doctoral studies very little was known about the disease and the search for resistance in *A. mangium* was proving challenging requiring a large number of different sources of germplasm to be screened and for alternative management strategies to be considered.

The aim of my first investigation was to improve screening procedures for resistance to *C. manginecans* in *Acacia* species by comparing the current screening protocol (inoculation of potted plants) with two potential rapid screening procedures; inoculation of detached acacia phyllodes (using a spore suspension) and stem segments (using mycelial plugs). Inoculation procedures were tested on three species of *Acacia*; *A. mangium*, *A. crassicarpa* and an *Acacia* hybrid (*A. mangium* x *A. auriculiformis* clones). Two cultures of *C. manginecans* obtained from different geographic regions were used in the experiments. Lesion length on stems and necrosis length on phyllodes were assessed and used as a measure of the susceptibility of the tested plant. The relative levels of susceptibility in each protocol were compared as a basis to assess the utility of the rapid screening protocols. The stem segments were prone to contamination by other fungi and to desiccation, while results from the potted plant and phyllode protocols showed similar trends of susceptibility among the *Acacia* clones and species.

The ease, rapidity and reproducibility of the phyllode inoculation protocol makes this a potential replacement for inoculation of potted plants as a preliminary screening protocol to identify *A. mangium* germplasm that is less susceptible to ceratocystis wilt and canker disease. We also evaluated whether the expression of resistance is influenced by the type of inoculum (mycelium or spore suspension). Both mycelial plugs and spore suspensions produced a similar level of disease incidence, so either can be used for inoculation assays permitting the testing of plants with spores of different isolates in the same inoculum. *Acacia crassicarpa* and *A. auriculiformis* are considered by the industry as promising sources for resistance against ceratocystis wilt and canker disease. This was also demonstrated in my study; the *A. crassicarpa* clones used were demonstrably more tolerant to *Ceratocystis* infection than *A. mangium*, with much smaller lesions. The disease indices for the *Acacia* hybrid were intermediate between *A. crassicarpa* and *A. mangium*.

Bacterial endophytes living in roots, stems and phyllodes of *A. mangium* were cultured to investigate their biodiversity and to obtain a library of isolates to test for biological control activity against *C. manginecans*. Samples from trees between one and five years old were collected and their culturable endophytic bacteria were identified by sequence analysis of their 16S rDNA. In total, 278 bacterial isolates were derived from 270 samples representing 90 trees. Most bacteria were isolated from roots and more isolates were obtained from young acacia trees than from the older trees. Analysis of 16S rDNA sequences grouped the endophytic bacteria into five clusters: Firmicutes, Alphaproteobacteria, Betaproteobacteria, Gammaproteo-bacteria and Actinobacteria. Firmicutes were predominant with 66.2% of the isolates belonging to this group. Among 25 genera which were isolated successfully, *Bacillus* and *Burkholderia* were the most frequently isolated endophytic bacteria in *A. mangium*. Several of the genera isolated from *A. mangium* are those previously demonstrated to include

species with potential as biological control agents (BCAs) against plant pathogens and/or the ability to enhance plant growth.

In-vitro assays of the 278 isolated endophytic bacteria against *C. manginecans* revealed 157 isolates with an ability to inhibit the growth of this fungal pathogen. Nine isolates demonstrated an ability to produce metabolites that inhibit the growth of *C. manginecans* in the absence of viable bacteria. The 16S rDNA analysis of these nine isolates placed them in the genera *Paenibacillus, Lysinibacillus, Staphylococcus, Pantoea, Ralstonia, Cupriavidus* and *Ochrobactrum.*

Several methods for inoculating the host tissue of *A. mangium* with these endophytic bacteria were evaluated. Two selected endophytic bacteria, *Paenibacillus* (EB-05/EB-93) and *Lysinibacillus* (EB-232) were introduced into *A. mangium* through the different methods and were positively detected in acacia tissues using specific primers. Applying liquid bacterial cultures to the root zone of germinating acacia seedlings was the most efficient inoculation method when compared with inoculation by seed treatment, or by dipping or spraying of acacia stem cuttings.

The deployment of any management strategy in the field will require monitoring to assess effectiveness. At the start of this thesis there was only anecdotal knowledge as to the timing of symptom development (e.g. reduction in leaf area index and yellowing) and the appearance of signs (e.g. insect damage) associated with *C. manginecans.* Healthy trees neighbouring diseased trees were monitored over 19 weeks. This data (especially LAI data) will be invaluable for carrying out monitoring activities, especially early detection through aerial observation.

In summary, the research in this thesis demonstrates the potential of phyllode inoculation as a rapid and low-cost method to extensively test and replicate resistance screening.

Selected bacterial endophytes inhibit *C. manginecans* isolates *in vitro* and these endophytes can be effectively delivered into a germinating *Acacia* seedling. The application of bacterial endophytes as biocontrol agents could be part of an alternative management strategy for *C. manginecans* integrated with the deployment of resistance. My study is the first attempt to establish a description and potential timeline associated with the disease life cycle. Such information can be used to improve monitoring programs for screening resistance and/or alternative management strategies in field experiments or the detection of ceratocystis wilt and canker disease for inventory purposes.

I. Introduction

I.1. Biotic threats to the sustainability of wood products from *Eucalyptus* and *Acacia* species in Indonesia

The global demand for paper products has led to an increase in pulp and paper industries worldwide over the last couple of decades. This industry is dominated by North American, Northern European and East Asian countries with a total production of 399 million tonnes of pulp and paper in 2012 which is expected to reach 490 million tonnes in 2020 (Bajpai 2013). Though the increasing use of electronic communications and data storage has previously led to predictions of a decline in the global demand for paper products, this trend, however, affects only the United States of America and Organization for Economic Co-operation and Development (OECD) countries, and had a negligible effect in Asia and developing regions, with increased demand in Africa and Latin America (Latta et al. 2015). This significant increment in paper and paperboard production has led to a very significant increase in the global demand for wood supply, from 280 to 500 million tonnes per annum. This demand is predicted to double again by 2030 (Obidzinski & Dermawan 2012).

In the Asia-Pacific, paper and paperboard production rose rapidly between 1980 and 2008, from 31 to 147 million tonnes (Asia-Pacific Forestry 2010). Indonesia is one of the countries in South-East Asia that has experienced especially dramatic increases in the production and trade of pulp and paper production (Barber 2004). Indonesian short-fibre pulp is exported to China, Korea, Europe, India, Japan and other Asian countries. The pulp production between 1988 and 2000 in Indonesia increased from 606,000 to 4.9 million metric tonnes and in the same period the production capacity of Indonesia's paper industry increased from 1.2 to 8.3 million tonnes per annum (Mohammed et al. 2012). By 2013 exports had

reached around 16.5 million tonnes (Wright 2014). As part of the need to place pulp and paper industries onto a more sustainable footing and become less reliant on sourcing pulpwood from native forest, Indonesia's Ministry of Forestry has promoted policies that encourage the development of a plantation-based wood supply (Obidzinski & Chaudhury 2009).

Maintaining a sustainable supply of the raw material for pulp and paper production is most often achieved by the extensive planting of exotic hardwoods and fast growing trees, particularly in tropical SE-Asia (Harwood & Nambiar 2014b). *Acacia* and *Eucalyptus* species are grown for pulpwood due to their potential for rapid growth, good quality wood and wide tolerance of various environmental conditions (Krisnawati et al. 2011). They are extensively planted in SE-Asia i.e. China, Indonesia, Vietnam, Malaysia, Thailand and Laos with 5-8 year rotations (Harwood & Nambiar 2014b). Three species currently account for most of the plantings in Indonesia because of their superior performance; *Acacia mangium* (~0.6M ha), *A. crassicarpa* (~0.3M ha) and *Eucalyptus pellita* and hybrids (>0.3 Mha) (Harwood & Nambiar 2014b). *Acacia mangium* has been preferred because of its good growth and satisfactory pulp yield under Indonesian conditions (Muhammad et al. 2018).

Despite their potential to produce large quantities of high-quality wood, there are several issues which threaten the sustainability of eucalypt and acacia pulpwood production. Species of *Acacia* and *Eucalyptus* are native to Australia, Papua New Guinea and eastern Indonesia and exotic to most regions where they are grown in extensive monocultures. Exotic trees can enjoy a honeymoon phase with very low disease levels when first planted in new areas due to the absence of their natural enemies. However, over time indigenous insects or pathogenic microorganisms may adapt to these exotic trees (Dark & Gent 2001). Insect pests and diseases from the tree's native range may also be moved with the exotic species or follow later (Wingfield et al. 2008). Both of these factors will lead to increased pest and disease incidence over time until these become a serious threat to the sustainability of their growth and

production. The causes of rapid colonization and outbreak of these insect pests and pathogens into new environments may be related to several factors i.e. the lack of the natural enemies, abundant food (monocultures), and the climatic and geographical environment (Weiss et al. 2019). Biosecurity in SE Asia is not always given priority and/or there is a lack of knowledge about potential threats. Seed and plant material may be indiscriminately moved between countries, with the potential transmission of damaging pests or microorganisms and any delay in response to new incursions greatly increases the risk of widespread establishment (Carnegie & Nahrung 2019).

Perhaps the most dramatic example of an emerging global threat to forest plantation health is *Leptocybe invasa*, a highly damaging eulophid gall wasp, which was unknown to science prior to its first report in Israel in 2000 (Mendel et al. 2004). Within approximately a decade, *L. invasa* expanded from native source populations in Australia to a minimum of 25 countries including South-East Asia and Indonesia, threatening the continued cultivation of numerous *Eucalyptus* species (Mendel et al. 2004; Garnas et al. 2012). Alongside other damaging Australian insect pests such as the leaf roller insect *Strepsicrates macropetana* and another gall wasp, *Ophelimus maskelli*, *L. invasa* was first seen causing significant damage in young eucalypts (Lawson 2007; Vastrad & Ramanagouda 2014) in New Zealand, China and Southeast Asia, including Indonesia, between 2002 and 2007 (Paine et al. 2011).

Bacterial wilt disease caused by the pathogen *Ralstonia solanacearum* is associated with significant mortality in eucalypts (Wingfield et al. 2008; Coutinho & Wingfield 2017), increasingly so in Indonesia. Bacterial wilt was first reported in eucalypt plantations in China (Cao 1982) and Brazil (Sudo et al. 1983), the centres of diversity of species of *Ralstonia*. Outbreaks of bacterial wilt in native stands or where most *Eucalyptus* species are native, such as in Australia have rarely been reported (Old et al. 2003). Bacterial wilt became problematic only when *Eucalyptus* were established in intensively managed plantations outside Australia

and the problem worsened when the industry moved toward clonal propagation from cuttings (Alfenas et al. 2006; Alfenas et al. 2009; Wang et al. 2014).

One of the two most important diseases currently confronted by Indonesian pulpwood industries using *Eucalyptus* and *Acacia* species is root-rot disease caused by facultative basidiomycete parasites. There are several candidate basidiomycete fungi that may act as primary or secondary root-rot pathogens in Malaysia and Indonesia (Glen et al. 2006). However, studies have identified *Ganoderma philippii* as the main species consistently associated with infected *A. mangium* roots displaying symptoms of red-root disease (Glen et al. 2009; Coetzee et al. 2011). In this case the fungal pathogen is indigenous. Mohammed et al. (2014) have reviewed root-rot diseases in tropical hardwood plantations and concluded that root-rot fungi are originally present in the native forest which when cleared for plantations are left in infected stumps and roots as inoculum sources. Disease build-up in woody debris left behind after harvest in short pulpwood rotations of five-to-seven years is associated with an accelerated development of disease such that tree death can exceed 50% in some areas within 20 years of establishing the first rotation.

The second devastating disease of plantation *Acacia* species in SE Asia is caused by a fungal pathogen belonging to the genus *Ceratocystis*. The fungal genus *Ceratocystis* includes many economically important tree pathogens (Kile 1993; Roux & Wingfield 2009; Harrington 2013). Until the 1980s, this genus of plant pathogens was not known from non-native plantation-grown forestry species (Roux & Wingfield 2009). However, over the past twenty years several reports have been made of *Ceratocystis* species causing death of non-native plantation-grown forestry species from several locations worldwide (Ribeiro et al. 1988; Morris et al. 1993; Laia et al. 1999; Roux et al. 2000b; Barnes et al. 2003a; Tarigan et al. 2011a; Thu et al. 2012; Li et al. 2014).

In SE Asia, a *Ceratocystis* species causes stem cankers and rapid wilting in several *Acacia* species and hybrids. Disease symptoms were first recognised approximately a decade ago in Indonesia and Vietnam, and *Ceratocystis* has also been reported as causing considerable losses in Malaysia. *Ceratocystis* isolates from infected *Acacia* were initially identified as *C. acaciivora* (Tarigan et al. 2011a), but later shown to be synonymous with *C. manginecans*, a serious pathogen of mango trees in Oman and Pakistan (Van Wyk et al. 2007; Tarigan et al. 2010; Fourie et al. 2015). The two species were at first differentiated by slight differences in their rDNA ITS sequence. Subsequent phylogenetic analyses showed that the ITS marker was unreliable (Al Adawi et al. 2013; Naidoo et al. 2013) and phylogenetic analyses based on four informative gene regions and single nucleotide polymorphism (SNP) markers, could not distinguish these two species (Fourie et al. 2015). The taxonomy of this group of fungi is, however, complex with some authors believing that *C. manginecans* is a synonym of *C. fimbriata* (Harrington et al. 2014; Oliveira et al. 2015) and work is ongoing to resolve species delimitations. In this thesis the fungal pathogen is referred to as *C. manginecans*.

Infection is often associated with pruning wounds such as singling wounds in *Acacia* species (Tarigan et al. 2011b) or other mechanical damage (monkeys and squirrels in *Acacia*). *Ceratocystis* species are vectored by wood-and bark-associated insects, typically those that visit fresh wounds acting as infection courts (Crone & Bachelder 1961; Moller et al. 1969; Wingfield et al. 1993; Roux et al. 2005; Heath et al. 2009a; Tarigan et al. 2011b). The fungi infect woody tissue and produce strong aromas (often like a brewery), which are attractive to insects (Lanza et al. 1976). Although it can be assumed that the biology of *C. manginecans* will be like other *Ceratocystis* species there have been no studies to confirm this or the significance of the different dissemination mechanisms usually associated with *Ceratocystis*.

Acacia mangium is particularly susceptible to both *Ganoderma* and *Ceratocystis*. Growth rates of *A. mangium* plantations in Sumatra ranged between 22 and 35 m^3/ha/yr before

being impacted by these fungal diseases, which have reduced growth on infected sites to 15 m^3/ha/yr or lower (Harwood & Nambiar 2014a).

At the start of my doctoral studies at the 2014 IUFRO Working Party 2.08.07: Genetics and Silviculture of Acacia International Conference in Vietnam the participants' conclusions to the management of pathogens and insect pests in *Acacia* species were:

- A sound understanding of basic science, epidemiology and ecology of every target organism is critical. Genetic solutions will be appropriate in many cases.
- No single solution is available and a belief in quick fixes can be counter-productive. Current research efforts in biological control show promise but need further field testing.

The clear message from the IUFRO conference was that there are many unresolved technical issues with acacia plantation forestry, and there is a need for a more integrated and inter-disciplinary approach to defining management systems which deliver sustainable and profitable plantation productivity. Effective disease management is critical to the economic viability of plantations in SE Asia. Switching from *Acacia* to *Eucalyptus*, the current reaction of large Indonesian pulp and paper companies, is not a universal solution. This is because, as explained above, *Eucalyptus* species have their own pest and disease challenges and site type limitations and require higher levels of management inputs (vegetation management and fertilizer application) to achieve satisfactory growth rates than do acacias.

I.2. Purpose of my research

The use of genetic resistance to limit disease impact has been proven cost effective, operationally feasible and sustainable in other major plantation forestry systems and was the first option investigated for *Ceratocystis* in *Acacia*. At the start of my doctoral studies the search for resistance in *A. mangium* was proving challenging (Brawner et al. 2015) requiring many different sources of germplasm to be screened. Little was known about the pathogen and

its variation in virulence. The catastrophic level of damage pushed for an immediacy of information, rapid screening preferably without transporting different strains of *Ceratocystis* to different areas of the plantation estate by carrying out inoculations in the field, and for alternative management strategies.

Current research into management strategies alternative to genetic approaches for *Ganoderma* in *A. mangium* focus on the application of bio-control agents especially antagonistic endophytes that can be cost-effectively inoculated in the nursery. The deployment of resistance and biological control are the only strategies likely to be deployed against insect pests and diseases with success over the many thousands of hectares involved in tropical plantations, especially in South-East Asia where topography, climate and cost often prohibit other management approaches such as chemical control or silvicultural options.

As in agriculture, remotely sensed digital imagery in forestry can be used to provide information with a higher level of coverage, consistency, spatial accuracy and automation of subsequent analysis on (1) the extent and progression of pest and disease outbreaks, (2) to predict susceptibility to specific damaging agents, and (3) as a tool for evaluating the performance of management strategies (Stone & Mohammed 2017). Knowledge of the phenology of the damaging agent and associated host response has implications for the optimal timing of data acquisition as well as the optimal reference data required for classifying or training the remotely sensed data (Stone & Mohammed 2017). While the symptoms and signs of *Ceratocystis* diseases are well described for other tree hosts, information about the symptom development (e.g. reduction in leaf area index and yellowing) and the appearance of signs (e.g. insect damage) associated with *C. manginecans* was largely anecdotal at the start of my doctoral studies.

My research therefore focused on (1) finding a rapid and cost-effective screening protocol for selecting resistant or tolerant *Acacia* germplasm against ceratocystis wilt and

canker disease; (2) identifying, evaluating and screening the potential ability of endophytic bacteria as BCAs against *C. manginecans* 3) testing the delivery of BCAs into *Acacia* germplasm; and (4) improving the knowledge about the development of crown and other symptoms and signs to better inform monitoring methodologies, especially those based on remote sensing.

I.3. Thesis structure

A literature review critically examines information supporting my approach to the research in this thesis. There are four experimental papers that cover the purpose of my thesis as set out above. A general discussion synthesises the results and discusses them in the context of both reducing losses from *C. manginecans* and the future management of insect pest and diseases which will undoubtedly increase with the expansion of exotic hardwoods in Indonesia and other countries of SE Asia.

My first paper is about finding a rapid screening protocol for the selection of tolerant and resistant acacia trees against ceratocystis wilt and canker disease. Two rapid screening procedures (inoculating acacia stem segments and phyllodes) were compared with the current screening protocol based on potted plant inoculation. Inoculation procedures were tested on three species of *Acacia*; *Acacia mangium, A. crassicarpa* and *Acacia* hybrid. Two cultures of *C. manginecans* obtained from different geographic regions were used in the experiments. Mycelial plugs were compared with spore suspensions as inoculum to infect artificial wounds on acacia seedlings.

My second paper describes the diversity of culturable endophytic bacteria isolated from *A. mangium* in Sumatra, Indonesia. Samples from trees between one and five years old were collected and their culturable endophytic bacteria were identified by sequence analysis of their 16S rDNA.

My third paper investigates the potential ability of endophytic bacteria to inhibit growth of the fungal pathogen *C. manginecans*. *In-vitro* assays tested 278 isolates of culturable endophytic bacteria. Several methods to deliver endophytic bacteria into acacia germplasm in the nursery are also explored in this paper.

The progression of symptoms and signs associated with ceratocystis disease in 2-year old *A. mangium* in Riau province, Sumatra, Indonesia is monitored in my fourth paper. Two separate areas in a plantation with ceratocystis disease were selected. Each area was 5 ha and approximately 5 km apart. Symptomatic *Acacia* trees were selected in both areas as well as eight asymptomatic trees adjacent to each symptomatic tree. A total of 135 trees were monitored over a 19-week period.

II. Literature Review

Acacia is a cosmopolitan genus of shrubs and trees which are native to Africa and Australia; the "genus" was first described by Phillip Miller in 1754 (Maslin et al. 2003a). However, this description was very broad and included several divergent lineages that needed to be placed in separate genera. A clear definition of the genus first emerged in the middle 19th century through several publications by George Bentham between 1840 and 1875 which classified *Acacia* into the subfamily Mimosoideae and categorized the plants as having numerous free stamens (Maslin et al. 2003a). The genus *Acacia* contains over 1350 species, distributed in tropical, subtropical and temperate regions including Europe, Africa, southern Asia, and the Americas (Maslin et al. 2003b; Orchard & Maslin 2003). However, phylogenetic studies have indicated that *Acacia* Miller *s.l.* is polyphyletic should be reclassified into five separate genera: *Acacia, Vachellia, Senegalia, Acaciella* and *Mariosousa* (Maslin 2008). Consequently, *Acacia* species in Africa have been grouped into two distinct genera, *Vachellia* and *Senegalia* (Haddad 2011; Kyalangalilwa et al. 2013), some of the American species are placed in genera *Acaciella* and *Mariosousa,* and approximately 960 species which are confined to Australia are still treated in the genera *Acacia* (Ebinger & Seigler 2005; Maslin 2008).

Acacia trees have been exploited for fuel wood, animal forage, human food, tannin and land rehabilitation (Midgley & Turnbull 2003). In southern and eastern Africa, they have been grown for more than 100 years for the control of sand drift, and for the timber, plywood and paper industries (Dunlop et al. 2003; Chan et al. 2015). In the Asia-Pacific region where large plantation estates have been established, some species have been grown for over 80 years. For example, the tropical *A. auriculiformis* was introduced from Australia to Malaysia in 1932, to Thailand in 1935 and subsequently to India and China (Midgley & Turnbull 2003). Two other tropical acacias, *A. mangium* and *A. crassicarpa* have been planted extensively in South-East

(S-E) Asian countries for at least three decades for pulpwood production (Griffin et al. 2011). *A. mangium* was first introduced from the humid tropical forests of north-eastern Australia to Malaysia in 1966 and subsequently to Papua New Guinea, Indonesia, Bangladesh, China, India, Philippines, Sri Lanka, Thailand and Vietnam (Midgley & Turnbull 2003; Krisnawati et al. 2011). *A. crassicarpa* was first planted in the early 1980s in Thailand and then also distributed to other countries, in particular in Indonesia where it is very well adapted to peatlands in Sumatra (Midgley & Turnbull 2003). A fourth species, *Acacia* hybrid (*A. mangium* x *A. auriculiformis*) was developed 20 years ago (Kha 2000) and is now the most commonly planted species in Vietnam (Griffin et al. 2011). All species are fast-growing, have good wood quality, are able to grow up to 30 m in height and can adapt to many types of soil and environmental conditions (Griffin et al. 2011; Krisnawati et al. 2011).

The utilization potential of these *Acacia* species for pulp and paper led to the rapid expansion of their planting and use by associated industries in S-E Asia. The total area planted in this region had reached over two million hectares by 2014 (Harwood & Nambiar 2014a), though by then, some estates reliant on *A. mangium* had already become non-commercial (Mohammed et al. 2014). This was because climate change and operational systems had combined to reinforce the threats of pathogens to forest production (Witzell et al. 2014). In particular, intensively-managed short-rotation monoculture plantations based on seedlings or clonal stock of a single species had affected forest biodiversity and led to the emergence of "new" diseases, often in the form of more aggressive strains of pathogens (Anderson et al. 2004; Martín-García et al. 2011).

In particular, wood yields and sustainability of production of *A. mangium* in Indonesia and Malaysia, have been compromised by two diseases. The first is red root-rot disease caused by *Ganoderma philippii* (Bres. & Henn. ex Sacc.) Bres. (Lee 2004; Coetzee et al. 2011; Francis et al. 2014; Mohammed et al. 2014). Acacias became infected when planted in areas where the

pathogen was already present, and losses in wood production increased with each rotation (Lee 2004; Francis et al. 2014). The second was wilt and canker disease caused by a species of *Ceratocystis* (Tarigan et al. 2011a; Fourie et al. 2015). The same pathogen is also a new disease threat in Vietnam (Thu et al. 2012). In Indonesia and Malaysia, this resulted in even greater mortality and loss of productivity than with root rot, and quickly led to the demise of this species as a source of pulpwood in Sumatra (Tarigan et al. 2011a; Brawner et al. 2015).

The focus of this literature review is on ceratocystis wilt and canker disease in acacia trees. Its aim is to synthesize information about this fungal pathogen that can be used to inform development of suitable disease-control strategies in forest plantations. However, an understanding of the origins and development of this disease requires reference to other species, particularly in the context of the biology and fungal taxonomy, disease symptoms and mechanisms of fungal dispersal. Some of the examples given below are no longer considered species of *Ceratocystis* due to recent taxonomic revisions (De Beer et al. 2017), but are still relevant in the discussion of disease transmission and control strategies. The risks and impacts of the disease on the sustainability of *Acacia* wood production and potential control strategies are then examined. Observing or surveying disease symptoms in plantations, selecting and planting tolerant or resistant *Acacia* trees, and the potential of endophytic bacteria as Biological Control Agents (BCA) also form part of this review.

II.1. Ceratocystis wilt and canker disease

Ceratocystis is a fungal genus with several species that cause rot diseases of agricultural crops and vascular wilt and canker stain of woody plants (Kile 1993; Roux & Wingfield 2009; Harrington 2013), though application of the term vascular wilt to diseases caused by *Ceratocystis* and related species is controversial (Kile 1993). A typical vascular wilt pathogen such as *Fusarium* or *Verticillium* moves through the xylem but does not invade xylem

parenchyma or ray cells until the host metabolism is disrupted and the tissues surrounding xylem vessels die (Talboys 1972). *Bretziella fagacearum* (Bretz) Z.W. de Beer, Marinc., T.A. Duong & M.J. Wingf. (syn. *C. fagacearum* (Bretz.) J. Hunt) is the only member of Ceratocystidaceae that fits this classical definition (Juzwik et al. 2008). By contrast, species such as *C. platani* (J.M. Walter) Engelbrecht & Harrington and *C. albifundus* M.J. Wingf., De Beer & M.J. Morris kill parenchyma tissue and also kill cambium and bark tissue, resulting in cankers (Morris et al. 1993; Lehtijärvi et al. 2018). *Ceratocystis* species invade their hosts through wounds, which may be caused by human activity, other mammals such as monkeys, elephants and squirrels, wind or boring insects (Kile 1993; Barnes et al. 2003b; Harrington 2007; Tarigan et al. 2011b).

The emergence of ceratocystis wilt and canker disease in commercial plantings of *Acacia* and also *Eucalyptus* spp. has been associated with a period of rapid growth of plantation estates based on these species following their introduction as non-native trees (Wingfield et al. 2001b; Roux & Wingfield 2009). Disease development is associated with discoloration of woody tissues, leaf yellowing, wilting and canker, and levels of mortality that affect the commercial viability of plantations (Barnes et al. 2003b; Roux & Wingfield 2009; Brawner et al. 2015). In acacias, these symptoms have been reported for *Acacia mearnsii* De Wild. in South Africa, Tanzania, Uganda (Morris et al. 1993; Roux et al. 2001a; Roux et al. 2005), for *A. mangium* in Indonesia (Tarigan et al. 2011a), Malaysia (Brawner et al. 2015) and Vietnam (Thu et al. 2012), and *A. decurrens* in Brazil (Ribeiro et al. 1988). These symptoms have also been reported for eucalypts in Africa (Roux et al. 2000b; Roux et al. 2001a), and South America (Laia et al. 1999; Barnes et al. 2003a). The incidence of ceratocystis disease in eucalypts in Indonesia is confined to a small number of susceptible clones (Heru Indrayadi, pers. comm.) and it has not yet been determined whether the pathogen on eucalypts is identical to that on *Acacia* though this work is in progress (Istiana Prihatini, pers. comm.).

II. 1.1. Causal pathogen

II.1.1.1. Taxonomy and plant hosts

The genus *Ceratocystis* and the genus *Ophiostoma* were previously placed in the same order, Ophiostomatales, based on morphological and ecological similarities. Ascocarps of both genera have a similar pattern of development and ecological niche; their necks are elongated and able to bear masses of sticky spores that easily stick to the legs and bodies of insects that feed on these fungi, facilitating spore dispersal to other trees (Malloch & Blackwell 1993). However, species in these genera can be distinguished through an examination of the anamorph (asexual) stage and their sensitivity to the antibiotic cycloheximide. *Ophiostoma* species are tolerant to cycloheximide, while *Ceratocystis* species are sensitive to cycloheximide (Samuels 1993). This difference and DNA sequence analysis of these genera has resulted in *Ceratocystis* and *Ophiostoma* being placed in different orders; *Ceratocystis* is now placed in the order Microascales (Spatafora & Blackwell 1994) and family *Ceratocystidaceae* (Réblová et al. 2011), distinct from Ophiostomatales.

A taxonomic revision of the family, supported by phylogenetic analyses (De Beer et al. 2014), resulted in the erection of two new genera, *Davidsoniella* and *Huntiella*, as well as emended descriptions for *Ambrosiella, Ceratocystis, Chalaropsis, Endoconidiophora* and *Thielaviopsis.* Species of *Ambrosiella* and *Endoconidiophora* are associates of ambrosia and bark beetles (Coleoptera: Scolytinae), with minimal direct impact on plant health. Most of the species pathogenic to dicotyledonous plants were placed into two of the emended genera, *Ceratocystis* and *Davidsoniella*, while pathogens of monocotyledonous plants fell into a single clade that corresponded to the emended description of *Thielaviopsis.* No sexual state is known for species retained or transferred to *Chalaropsis*, and the asexual state is indistinguishable from those of *Ceratocystis* spp. These species are found on woody substrates but are not known

to have any ecological or economic significance (De Beer et al. 2014). *Huntiella* includes wound-colonising saprobes or mild pathogens that may be responsible for sap stain in timber. Some species, including the oak wilt pathogen *C. fagacearum,* did not fit into a well-defined clade. Subsequently another new genus, *Bretziella,* was erected to accommodate *B. fagacearum,* syn. *Ceratocystis fagacearum* (De Beer et al. 2017).

The species remaining in the redefined genus *Ceratocystis* consist of those conforming to the previous species concept of *C. fimbriata* Ellis & Halst. (De Beer et al. 2014; Liu et al. 2018). Species delineations in this group are controversial and some authors maintain that many of the newly described species are conspecific with *C. fimbriata* (Oliveira et al. 2015).

The pathogenic association of a species of *Ceratocystis* with a cultivated crop was first reported in 1890 in New Jersey, USA, where it was associated with tuber black rot on *Ipomoea batatas* (L.) Lam. (sweet potato) (Halsted & Fairchild 1891). The causal fungus was described as the new species *C. fimbriata*. Since then, a wide range of agricultural crops and woody trees, both gymnosperms and angiosperms, has been reported as hosts of *Ceratocystis fimbriata* and related species (Harrington 2004; Roux & Wingfield 2009; Al Adawi et al. 2013). Baker et al. (2003) list 31 species of plants from 14 families as hosts of *Ceratocystis* spp. As well as many tree species, this includes root crops, edible aroids (*Araceae*), *Coffea arabica* L. and *Theobroma cacao* L. In *Colocasia esculenta* (L.) Schott (taro), *C. fimbriata* causes a post-harvest rot, similar to that on sweet potato (Harrington et al. 2015).

Tree hosts such as, *Ficus carica* L., *Terminalia ivorensis* A.Chev., *Prosopis cineraria* (Linn.), *Dalbergia sissoo* Roxb. ex DC., *Platanus* spp., *Eucalyptus* spp., *Acacia* spp., *Hevea brasiliensis* Müll. Arg., and other cultivated trees are also affected (Kile 1993; Harrington 2007; Al Adawi et al. 2013; De Beer et al. 2014). Economically important levels of damage can be caused, for instance tree death of *Mangifera indica* L. (mango) caused by *Ceratocystis manginecans* M. van Wyk, Al Adawi & M.J. Wingf. (Huang et al. 2003), and wilt disease of

Punica granatum L. (pomegranate) by *C. fimbriata* (Huang et al. 2003). Environmental impacts of *Ceratocystis* species may also be dramatic; for example, rapid ohia death (ROD) in *Metrosideros polymorpha* Gauch. (Keith et al. 2015).

Initially identified as *C. fimbriata* (Morris et al. 1993; Roux & Wingfield 2009) several new *Ceratocystis* species have been described as causing serious damage on *Acacia* spp., for example *C. albifundus* wilt and canker disease in *A. mearnsii* (Morris et al. 1993; Roux et al. 1999; Barnes et al. 2005). The *Ceratocystis* species infecting *Acacia* spp. in Indonesia was initially identified as belonging to two species, *C. manginecans*, originally described from *M. indica* in Oman (Van Wyk et al. 2007), and a new species, *C. acaciivora* Tarigan & M. van Wyk (Tarigan et al. 2010), the two being differentiated by slight differences in their rDNA ITS sequence. Subsequent phylogenetic analyses showed that the ITS marker was unreliable (Al Adawi et al. 2013; Naidoo et al. 2013) and phylogenetic analyses based on four informative gene regions and single nucleotide polymorphism (SNP) markers, could not distinguish these two species (Fourie et al. 2015). As a result *C. acaciivora* is considered synonymous with *C. manginecans* (Fourie et al. 2015). *Ceratocystis manginecans* infects *Lansium parasiticum* (Osbeck) K.C. Sahni & Bennet (duku) as well as *Acacia* spp. in Indonesia (Irsan et al. 2016), though has not been reported from mango in Indonesia. In Oman and Pakistan it infects native legume trees (Al Adawi et al. 2013) as well as mango (Van Wyk et al. 2007) (Table II-1). Two new species, *C. mangicola* M. van Wyk & M.J. Wingf. and *C. mangivora* M. van Wyk & M.J. Wingf., were described from mango in Brazil (Van Wyk et al. 2011), however pathologists in Brazil continue to regard the pathogens on mango, *Eucalyptus, Hevea, Tectona* and many other host species as members of a single species, *C. fimbriata,* (Firmino et al. 2012; Harrington et al. 2014; Valdetaro et al. 2015; Oliveira et al. 2016). Recent population genetic studies provide some evidence to support this view. Isolates from kiwifruit (*Actinidia* spp.) separated into three distinct groups, with one group closely related to isolates from Eucalypts and another closely

related to isolates from mango and taro (Ferreira et al. 2017). The majority of isolates belonged to a third group, labelled PM, that was linked to the nursery that supplied kiwifruit plants to the other sampled farms. The high level of clonal replication indicated a strong likelihood of vegetative reproduction, either in infected scion material or by contaminated tools. The relative pathogenic aggressiveness of isolates from the three groups was not tested, though all groups were isolated from multiple diseased kiwifruit vines. Phylogenetic analysis of the mating type genes provided additional support for groupings based on microsatellite data (Ferreira et al. 2017), with the PM population the most divergent.

II.1.1.2. Morphology and reproduction

Ceratocystis spp. have sexual and asexual stages of reproduction. The sexual stage can be recognised through the presence of small, dark- through to light-coloured, sub-globose, globose or spherical fruiting bodies known as ascocarps (or ascomata) which are typically ostiolate or perithecial. The base of the ascocarps is enlarged and they have long necks with ostiolar hyphae on the tip (Figure II-1, cited from De Beer et al. (2014)). Deliquescent asci emerge from the centrum of the ascocarp and produce sticky, hat-shaped ascospores (sexual spores) (Upadhyay 1993). The ascospores are hyaline and lack germ-pores. The ascospores exude from the ascocarp as a sticky droplet through the ostiolar hyphae at the top of the ascomatal necks. These spores adhere easily to insects and this may assist their dispersal (Upadhyay 1993; De Beer et al. 2014).

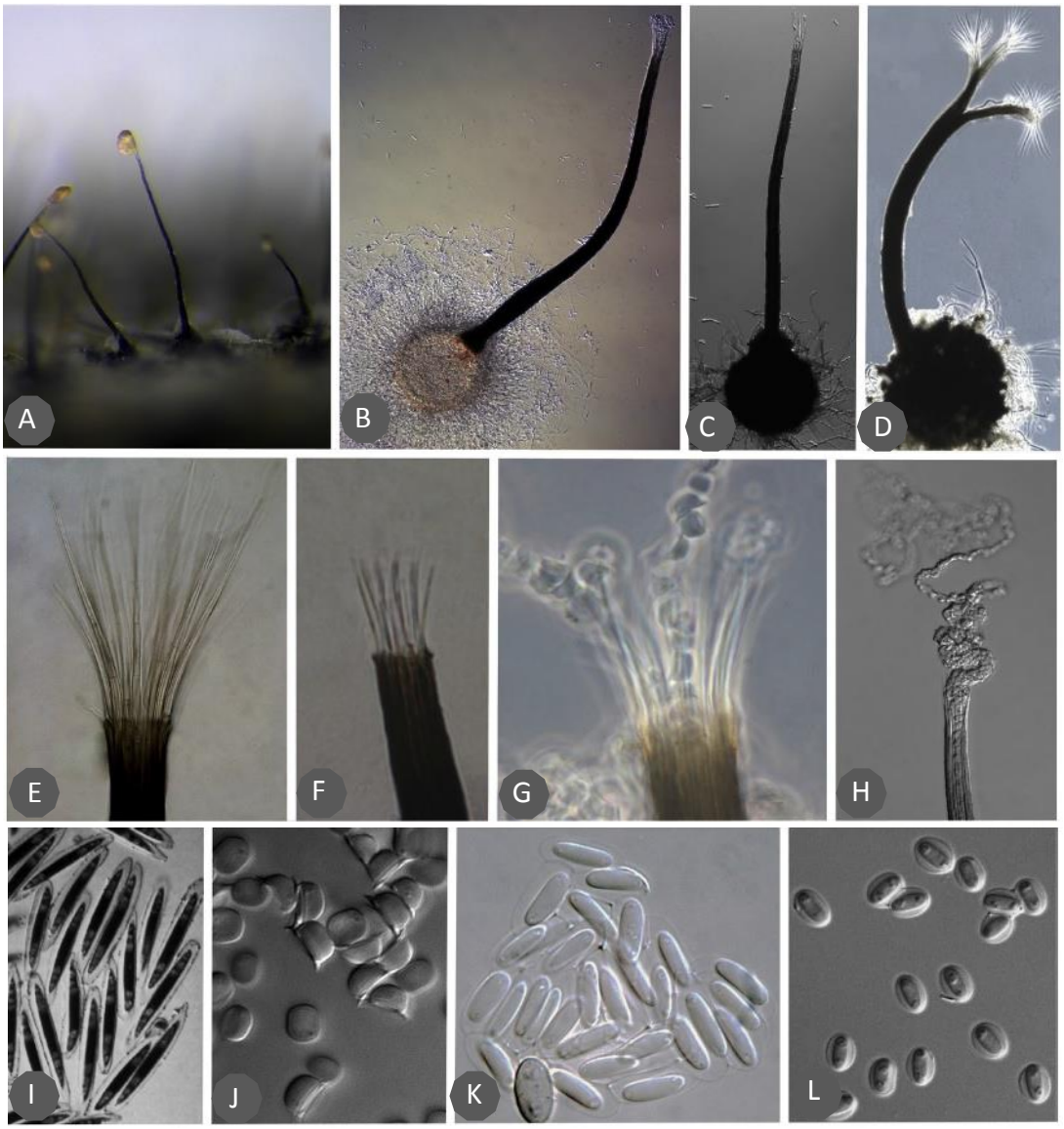

Figure II-1. Ascomatal morphology of *Ceratocystis fimbriata* with long necks and ostiolar hyphae (A), some morphological features of ascomata; light-coloured bases of *C. albifundus* (CMW4059) (B), pear-shaped bases of *C. pirilliformis* (CMW6579) (C) and ornamented bases and divergent necks of C. *cerberus* (CMW 36668) (D). E to F show the forms of ostiolar hyphae where the ascospore masses appear; long and divergent (E), short and convergent (F). The ascospores (teleomorph spores) are released from ostiolar hyphae (G-H) and I to L show the shapes of ascospores, fusoid (I), hat-shaped (J), oblong (K), and obovoid (L). (Photo cited from De Beer et al. (2014)).

The anamorph stage of *Ceratocystis* is morphologically like that of *Chalara* or *Thielaviopsis* species forms. All *Ceratocystis* species have a *Chalara* or *Thielaviopsis*-like anamorph (Paulin-Mahady et al. 2002; Harrington 2013; De Beer et al. 2014). Simple tubular conidiogenous cells called phialides typically taper towards their apices, and produce either chains of rectangular conidia or dark barrel-shaped secondary conidia as asexual spores (De Beer et al. 2014). Production of aleuroconidia by some species of *Ceratocystis* facilitates survival in and transmission through soil. These asexual spores are characterized by pigmented, thick-walled, chlamydospore-like spores and produced through particular conidiophores (Figure II-2, cited from De Beer et al. (2014)) (Harrington 2013).

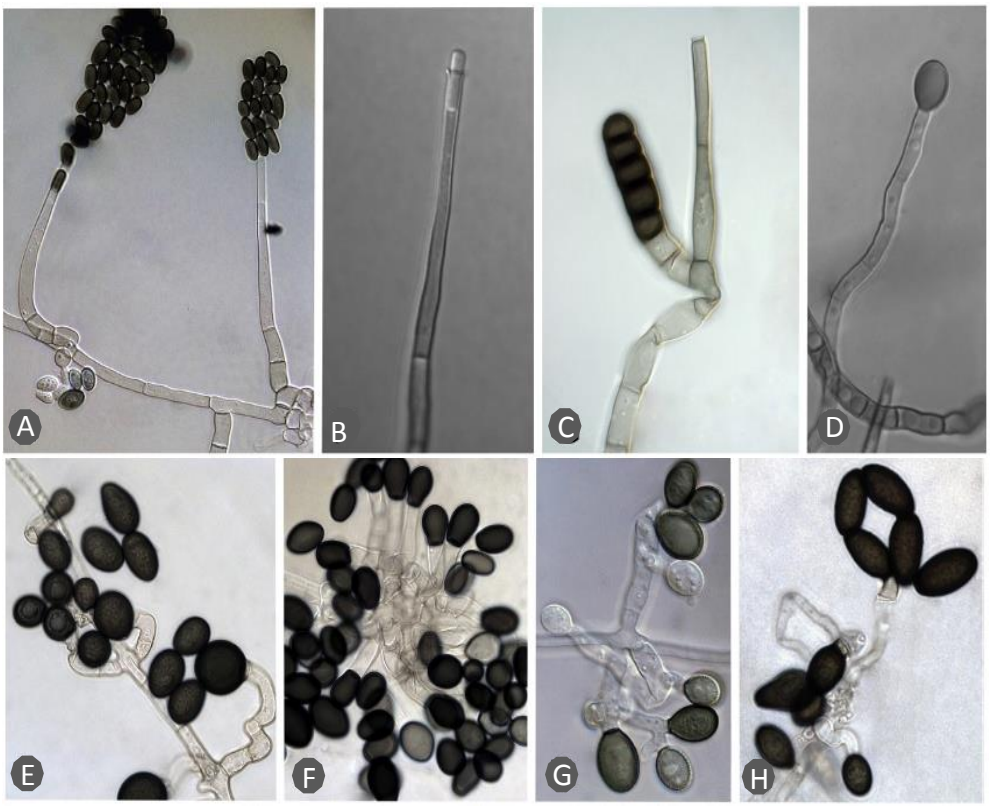

Figure II-2. Anamorph spores of *Ceratocystis* sp. Conidia with several forms are released from phialidic conidiophores; obovoid conidia are released from simple tubular conidiophore (A), phialide releasing cylindrical conidia (B), chlamydospores of *Berkeleyomyces basicola* (C) and *Ceratocystis pirilliformis* (D). The darkly pigmented and thick walled aleurioconidia are shown in E to H. (Photo cited from De Beer et al. (2014)).

II.1.1.3. Geographic distribution of *Ceratocystis* spp. infecting woody hosts with particular reference to biosecurity for Australian hardwoods

Most species of *Acacia* and *Eucalyptus* are endemic to Australia though several species of both genera have been adopted for hardwood plantations in other countries. This widespread deployment in other countries exposes these species to 'new encounter' pests and pathogens (Wingfield et al. 2011). *Austropuccinia psidii* and *Ceratocystis fimbriata s.l.* are two examples of pathogens that have moved from native hosts onto eucalypts in Brazil (Zauza et al. 2004; Glen et al. 2007). Such pathogens represent a biosecurity threat to Australia. It is, therefore, critical for Australian biosecurity to understand the species identity and host ranges of these pathogens to inform risk and pathway analyses. Likewise, given the impact that *Ceratocystis manginecans* can have on acacias and other tree species including mango, a thorough understanding of the identity, including any taxonomic uncertainties, geographic and host ranges of *Ceratocystis* species infecting woody plants would be beneficial to Australian biosecurity preparedness. The willingness to assign a new species name to any group of isolates/specimens that can be discriminated by any DNA synapomorphy , however obscure, combined with the lack of knowledge on the genetic basis of host specificity in *Ceratocystis,* confounds the issue as the new names are not universally accepted (Oliveira et al. 2015) and new disease reports still ascribe disease in woody plants to *C. fimbriata* (Alam et al. 2018; Xu et al. 2019). Several of the newly described species can only be discriminated by DNA sequence data from carefully selected genomic regions and/or microsatellite analysis (Liu et al. 2018), a technique more commonly applied to population genetic analysis of a single species. This section provides examples of the confusion surrounding the geographic and host ranges of *Ceratocystis* species that infect woody plants, with a focus on those causing disease in acacias and eucalypts.

The genus *Ceratocystis* has a wide geographic distribution and can be found causing serious disease to a range of species in tropical, subtropical and temperate climates and in all continents (Table II-1). In the previous century, many reports of damage to woody hosts were ascribed to *C. fimbriata.* Many of these pathogens have subsequently been described as new species, e.g. wilt disease in *A. mearnsii* in South Africa was initially ascribed to *C. fimbriata* (Morris et al. 1993) and later described as *C. albifundus* (Wingfield et al. 1996).

Ceratocystis species may be native to a specific environment and disperse to other geographic regions, where the impact may be greater than in its native range. For instance, *C. pirilliformis* has been recorded from Australia, where it causes sap-stain in *Eucalyptus nitens* (H. Deane & Maiden) Maiden (Barnes et al. 2003b) but has not been reported in association with *Acacia* spp. A molecular and phylogenetic study using polymorphic simple sequence repeat (SSR) markers on *C. pirilliformis* indicated that this pathogen is more diverse in Australia than in South Africa and therefore presumed to be native to Australia (Nkuekam et al. 2009). In South Africa it causes vascular stain on *E. grandis* W. Hill (Barnes et al. 2003b; Nkuekam et al. 2009) in addition to *A. mearnsii*. Two other species, *C. atrox* M. van Wyk & M.J. Wingf. and *C. corymbiicola* Kamgan-Nkuek., occur on eucalypts in Australia, while another six have been described from *Eucalyptus* spp. in South Africa and South America (Table II-1).

The most severe outbreaks of *Ceratocystis* wilt and canker of *Acacia* species have been in S-E Asia where *C. manginecans* infects *Acacia* species including *A. auriculiformis, A. crassicarpa* and *A. mangium* (Tarigan et al. 2010; Thu et al. 2012; Brawner et al. 2015). This species was first described from mango trees in Oman and Pakistan (Van Wyk et al. 2007). Other hosts include *Dalbergia sissoo* DC. in Pakistan (Al Adawi et al. 2013), *Lansium parasiticum* (Osbeck) K.C. Sahni & Bennet in Indonesia (Irsan et al. 2016), *Mimusops elengi* L. in Thailand (Pornsuriya & Sunpapao 2015) and *Prosopis cineraria* (L.) Druce in Oman (Al

Adawi et al. 2013). Infections of *Eucalyptus* spp. also occur in Indonesia, but the species identification has not yet been confirmed (Indrayadi, pers. comm.). However, recently in Pakistan the pathogen which infects *Eucalyptus* spp. has been identified as *Ceratocystis fimbriata* (Alam et al. 2017) based on culture morphology and DNA sequencing of the rDNA ITS region. The rDNA ITS is not considered adequate for discrimination of *Ceratocystis* species (Oliveira et al. 2015; Liu et al. 2018). Thus, it remains unclear whether the isolates that infect eucalypts, loquat and pomegranate in Pakistan are con-specific with those that infect mango and *Dalbergia* in Pakistan.

Many of the recently described *Ceratocystis* species lived in equilibrium with indigenous hosts in their native environment and attracted little scientific attention until they moved onto a susceptible, exotic host species in the same or another country (Baker et al. 2003). Subsequent isolation and DNA sequencing enabled identification as novel species (Barnes et al. 2003b), for example *Ceratocystis fimbriotomima* M. van Wyk & M.J. Wingf. in Venezuela that infected introduced *Eucalyptus* spp. as well as native species (Van Wyk et al. 2009). Similarly, *C. colombiana* M. van Wyk & M.J. Wingf. that infects native trees in Colombia was identified as a new pathogen infecting introduced coffee, cacao and citrus (Van Wyk et al. 2010), and *C. ficicola* Kajitani & Masuya that infects native trees in Japan, as a new pathogen that infects *Ficus drupacea* Thunb. (syn. *Ficus indica* L.) (Kajitani & Masuya 2011). Morphologically these three species are very close to *C. fimbriata*; support for their separation into distinct taxa depends on comparison of carefully selected DNA sequence data (Van Wyk et al. 2010; Kajitani & Masuya 2011).

Discrimination among many *Ceratocystis* species is highly dependent on an enthusiastic application of phylogenetic species recognition concepts where every grouping that forms a well-supported clade is deemed to be a separate species. There is a risk that this approach may identify geographically or environmentally isolated sub-populations as distinct

species (Harrington et al. 2014; Fourie et al. 2015). While these new 'species' are shown to be distinct, host-adapted lineages, it is not clear whether they fit the classical biological species concept (Oliveira et al. 2015). Fungi with both sexual and asexual methods of reproduction can have complicated population genetics that may lead to confusion over species boundaries, e.g. *C. acaciivora*. The internal transcribed spacer region (ITS) is commonly used for phylogenetic analyses, but this region can produce misleading results in *Ceratocystis* species; a single haploid isolate has been shown to contain two widely divergent ITS sequences (Harrington et al. 2014).

Mating studies have confirmed species boundaries among *C. fimbriata s.s.*, *C. cacaofunesta* and *C. platani* (Engelbrecht & Harrington 2005), with interspecific pairings mainly infertile, though some produced perithecia either lacking ascospore masses or with transparent or milky ascospore masses and the few, if any, ascospores observed were misshapen. Few 'interspecific' mating tests have been conducted in *Ceratocystis*, but success in mating *C. manginecans* with *C. fimbriata s.s.* has been demonstrated (Fourie et al. 2018). Of the 70 hybrid offspring produced, eleven were pathogenic to sweet potato, seven were pathogenic to *A. mangium* and three were pathogenic to both. The authors concluded that host specificity was governed by a small number of genes.

Table II-1. *Ceratocystis* spp. on woody plants, their associated host species and geographic locations.

***Ceratocystis* species**	**Host**	**Geographic location**	**References**
C. albifundus	*Acacia caffra*	South Africa	Roux et al. (2007)
	A. decurrens	South Africa	Roux and Wingfield (2009)
	A. mearnsii	South Africa, Uganda Tanzania, Kenya Malawi	Roux et al. (2001b) Roux et al. (2005) Roux et al. (2004a)
	A. nigrescens	South Africa	Kamgan et al. (2008)
	Dahlbergia nitidula	Zambia	Roux and Wingfield (2013)
	Protea spp.	South Africa	Roux et al. (2007)
	Terminalia sericea	South Africa	Roux et al. (2007)
	Ochna pulchra	South Africa	Roux et al. (2007)
	Burkea africana	South Africa	Roux et al. (2007)
	Faurea saligna	South Africa	Roux et al. (2007)
	Ozoroa paniculosa	South Africa	Roux et al. (2007)
C. atrox	*Eucalyptus grandis*	Australia	Van Wyk et al. (2007)
C. cacaofunesta	*Theobroma cacao*	Brazil, Columbia, Costa Rica, Ecuador, Guatemala, Trinidad & Tobago, Venezuela	Engelbrecht et al. (2007a)
	Herrania sp.	Costa Rica	Engelbrecht et al. (2007b)

Table II-1. *Ceratocystis* spp. on woody plants, their associated host species and geographic locations (continued)

***Ceratocystis* species**	**Host**	**Geographic location**	**References**
C. caryae	*Carya* spp.	North America	Johnson et al. (2005)
C. cercfabiensis	*Eucalyptus* spp. (stumps)	South China	Liu et al. (2015)
C. collisensis	*Cunninghamia lanceolata*	South China	Liu et al. (2015)
C. colombiana	*Citrus* spp., *Coffea* sp.	Colombia	Van Wyk et al. (2010)
	Coffea arabica	Colombia	Van Wyk et al. (2010)
	Schizolobium parahybum	Colombia	Van Wyk et al. (2010)
C. corymbiicola	*Corymbia variegata*	Australia	Nkuekam et al. (2012)
	Eucalyptus spp.	Australia	Nkuekam et al. (2012)
C. curvata	*Eucalyptus deglupta*	Colombia, Ecuador	Van Wyk et al. (2011)
C. diversiconidia	*Terminalia ivorensis*	Colombia, Ecuador	Van Wyk et al. (2011)
C. ecuadoriana	*Eucalyptus deglupta*	Colombia, Ecuador	Van Wyk et al. (2011)
C. eucalypticola	*Eucalyptus* spp.	South Africa	Nkuekam et al. (2013)
	Eucalyptus sp.	Republic of the Congo, Uganda	van Wyk et al. (2012)
		Brazil	Ferreira et al. (1999)
C. ficicola	*Ficus carica*	Japan	Kajitani and Masuya (2011)
C. fimbriatomima	*Eucalyptus* sp.	Venezuela	Van Wyk et al. (2009)

Table II-1. *Ceratocystis* spp. on woody plants, their associated host species and geographic locations (continued)

***Ceratocystis* species**	**Host**	**Geographic location**	**References**
C. fimbriata	*Ipomoea batatas*)*	Brazil, China, Cost Rica, Haiti, Japan, Kenya, Korea, Mexico, New Zealand, Japan, Kenya, Korea, Papua New Guinea, Puerto Rico, Taiwan, USA, Virgin Is., West Indies	USDA ARS
C. harringtonii	*Populus* spp.	Canada, USA, Poland	Johnson et al. (2005)
C. huliohia	*Metrosideros poymorpha*	USA (Hawaii)	Barnes et al. (2018)
C. larium	*Styrax benzoin*	Indonesia	Van Wyk et al. (2009)
C. lukuohia	*Metrosideros polymorpha*	USA (Hawaii)	Barnes et al. (2018)
C. mangicola	*Mangifera indica*	Brazil	Van Wyk et al. (2011)
C. manginecans	*Acacia crassicarpa*	Indonesia (Sumatra)	Al Adawi et al. (2013)
	Acacia mangium	Indonesia, Malaysia, Vietnam	Tarigan et al (2011a); Brawner (2015); Thu et al. (2012)
	Dalbergia sissoo	Pakistan	Al Adawi et al. (2013)
	Lansium parasiticum	Indonesia	Irsan & Suwandi, Sriwijaya University (unpublished)
	Mangifera indica	Oman, Pakistan	Van Wyk et al. (2007)
	Mimusops elengi	Thailand	Pornsuriya and Sunpapao (2015)
	Prosopis cineraria	Oman	Al Adawi et al. (2013)

Table II-1. *Ceratocystis* spp. on woody plants, their associated host species and geographic locations (continued)

***Ceratocystis* species**	**Host**	**Geographic location**	**References**
C. mangivora	*Mangifera indica*	Brazil	Van Wyk et al. (2011)
C. neglecta	*Eucalyptus grandis*	Colombia	Rodas (2008)
C. obpyriformis	*Acacia mearnsii*	South Africa	Heath et al. (2009b)
C. papillata	*Annona muricata*	Colombia	Van Wyk et al. (2010)
	Citrus spp.	Colombia	Van Wyk et al. (2010)
	Coffea arabica	Colombia	Van Wyk et al. (2010)
	Schizolobium parahybum	Colombia	Van Wyk et al. (2010)
	Theobroma cacao	Colombia	Van Wyk et al. (2010)
C. pirilliformis	*Acacia mearnsii*	South Africa	Lee et al. (2016)
	Eucalyptus spp.	South Africa, Malawi	Nkuekam et al. (2013)
	Eucalyptus spp.	Australia	Barnes et al. (2003b)
	Rapanea melanophloeos	South Africa	Lee et al. (2016)
C. platani	*Platanus* spp.	Albania, France, Italy, Spain, USA	Tsopelas et al. (2017)
	Platanus spp.	Greece, Italy, Switzerland, Turkey	Lehtijärvi et al. (2018)
	Syngonium podophyllum	USA (Hawaii)	Li et al. (2017)
C. polychroma	*Syzygium aromaticum*	Indonesia (Sulawesi)	Van Wyk et al. (2004)
C. polyconidia	*Acacia mearnsii*	South Africa	Heath et al. (2009b)
C. smalleyi	*Carya* spp.	USA	Johnson et al. (2005)

Table II-1. *Ceratocystis* spp. on woody plants, their associated host species and geographic locations (continued)

***Ceratocystis* species**	**Host**	**Geographic location**	**References**
C. tanganyicensis	*Acacia mearnsii*	Tanzania	Heath et al. (2009b)
C. thulamelensis	*Colophospermum mopane*	South Africa	Mbenoun et al. (2014)
	Combretum zeyheri	South Africa	Mbenoun et al. (2014)
C. tsitsikammensis	*Acacia melanoxylon*	South Africa	Misse et al. (2017)
	Eucalyptus sp.	South Africa	Misse et al. (2017)
	Ocotea bullata	South Africa	Kamgan et al. (2008)
	Rapanea melanophloeos	South Africa	Kamgan et al. (2008)
	Terminalia sericea	South Africa	Kamgan et al. (2008)
C. variospora	*Betula platyphylla*	Japan	Linnakoski et al. (2008)
	Prunus sp.	USA	Li et al. (2017)
	Quercus spp.	USA	Johnson et al. (2005)
C. zambeziensis	*Acacia nigrescens*	South Africa	Mbenoun et al. (2014)
	Combretum imberbe	South Africa	Mbenoun et al. (2014)
	Schotia brachypetala	South Africa	Mbenoun et al. (2014)

*)*Ceratocystis fimbriata s.l.* has been reported from nearly 100 host species including many woody plants, so would require a complete table of its own. All species in this table were previously encompassed by *C. fimbriata* and some of the newly delineated species are not universally accepted as distinct from *C. fimbriata*, which was first described from sweet potato. Isolates from sweet potato do not infect woody hosts though it is unclear whether or not this represents a species boundary or a host-specialised form (Valdetaro et al. 2015).

II. 1.2. Disease

II.1.2.1. Symptoms

Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *Ceratocystis* species (Kile 1993). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *Ceratocystis* species in woody trees (Kile 1993; Roux & Wingfield 2009; Harrington 2013). Tuber crops such as *I. batatas* and *C. esculenta* commonly exhibit black rot symptoms (Muramoto et al. 2012; Harrington et al. 2015).

The sudden wilting of leaves is the earliest visible symptom associated with true vascular wilt. In this case, the pathogen travels through the non-living water-conducting vessels and tracheids, colonizing the host away from the wound. An example is *B. fagacearum* that attacks *Quercus* species (Kile 1993). This wilting develops as the hyphae grow through and then plug the vessels, blocking the conducting system above the site of infection, thereby desiccating the plant (Mace et al. 1981).

By contrast, in *Ceratocystis* infections, sapwood discoloration is induced after the pathogen attacks living parenchyma cells (Harrington 2007). Staining or sap streaks can start from where spores have invaded freshly damaged tissue, they rapidly germinate and colonise the xylem and phloem (Johnson et al. 2005), absorbing nutrients from the xylem parenchyma (Mace et al. 1981). The discoloration is caused by a combination of host response chemicals and the pigmentation of the spores and hyphae of *Ceratocystis* species (Harrington 2013). Once present in the vascular cylinder, the hyphae of *Ceratocystis* species can then move systematically into the cambium and inner bark; killing these tissues and causing a canker (Kile 1993; Harrington 2013).

In Acacias, *Ceratocystis* species cause vascular stain, canker on the stem and wilting. Once infected, the symptoms are first expressed as black or red lesions on the bark, dark streaks within vascular tissue or sapwood discoloration. Cankers on the stem and cracked or sunken bark above cankers emerge as further symptoms. This is followed with yellowing leaves, wilting, and death of the tree due to lack of nutrient supply into the plant (Roux et al. 2001b; Tarigan et al. 2011a; Brawner et al. 2015). The foam or fermentation exudate of yeasts or bacteria also often emerge from the lesions or from entrance holes made by stem borer or fungal feeding insects near to stem cankers (Figure II-3). In particular, this exudate attracts fungivorous nitidulid beetles (Coleoptera: *Nitidulidae*) which are associated with fungal dispersal (Brawner et al. 2015).

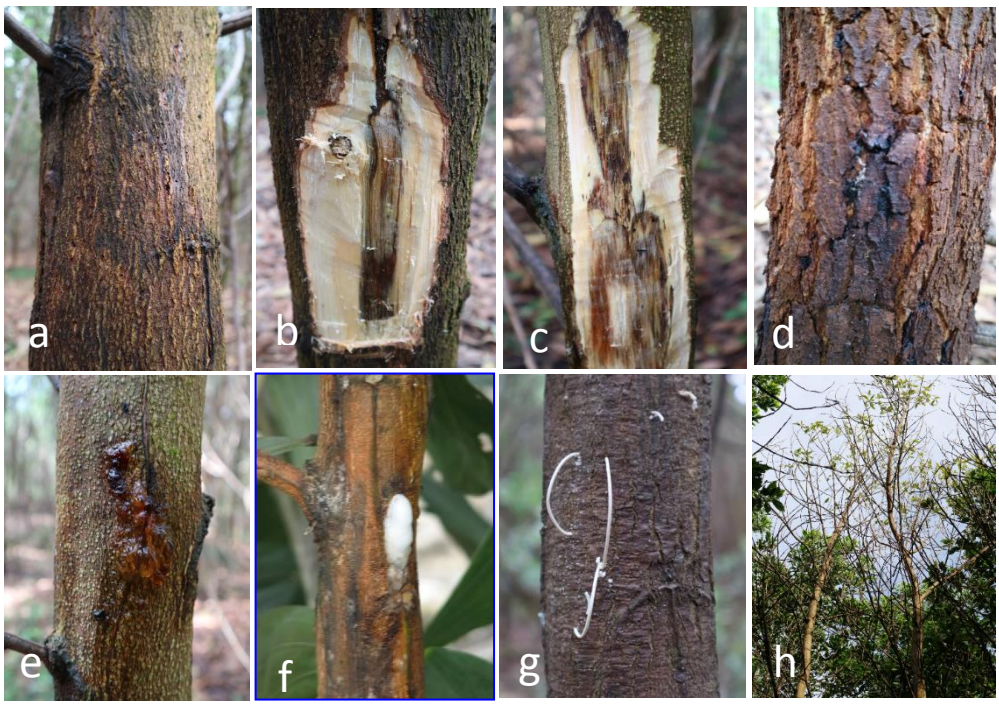

Figure II-3. Ceratocystis wilt and canker disease symptom on *Acacia mangium*. External discoloration (a), internal discoloration (b,c), canker on stem (d), gummosis (e), fermented exudate with fruity odours (f), frass exuded by stem-boring insects (g) and yellowing leaves and die back of tree (h) (Aswardi Nasution, 2017).

II.1.2.2. Epidemiology

Disease spread depends upon inoculum production and dispersal, the presence of susceptible hosts and suitable environmental conditions. *Ceratocystis* species produce several kinds of spore inoculum and exploit a range of dispersal mechanisms which may vary according to the type of spores produced. Like many filamentous fungi, the spores of *Ceratocystis* species infect their hosts through an infection court, often a wound, where the spores germinate after deposition (Kile 1993; Harrington 2013); wounding is therefore critical for infection to occur. Root grafting may also contribute to disease development (Harrington 2013).

For woody trees, the most common mechanism of disease spread is through spore dispersal, vector activity, and mechanical transmission. Spore types may include endoconidia, aleurioconidia, chlamydospores (formed by thickening of the walls of endoconidia) and ascospores. The first three are asexual spores while ascospores are the products of sexual recombination. Sporulating mats on exposed wood or in the bark of infected trees produce all spore types (Friday et al. 2016) while aleurioconidia are particularly abundant in the infected wood (Araujo et al. 2014). Conidia and ascospores have a short period of viability whereas aleurioconidia have thicker, more durable walls and can likely survive for years, especially inside wood, and increase the likelihood of the disease being soil-borne (Paulin-Mahady et al. 2002).

Aleurioconidia are typically soil-borne, though may also be present in insect frass which is ejected from trees and wind-dispersed (Souza et al. 2013). Species of *Ceratocystis* produce aleurioconidia and are known soil-borne pathogens (Harrington 2007). The thick-walled aleurioconidia can survive for years in the soil (Upadhyay 1981). Subsequent germination then has the potential to infect new plant material brought into the area (Moutia & Saumtally 2001; Marin Montoya et al. 2003). The status of *C. manginecans* as a soil-borne

pathogen is unclear. It has been isolated from soil in Vietnamese acacia plantations (Thu et al. 2016), and attempts to isolate from soil in Indonesian plantations have recently been successful (Marthin Tarigan, pers. comm.), though DNA sequencing is yet to confirm accurate identification of the soil isolates. Ascospores produced by fruiting bodies on woody stems or branches may be transmitted through an insect vector, mechanical tools, wind and water (Kile 1993).

Disease spread can also occur via natural root grafts between infected and healthy roots, a phenomenon that occurs in *Quercus rubra* L. and that facilitates the transmission of *B. fagacearum* without first passing through the root collar (Juzwik et al. 2008). This same pathogen was isolated from around one-quarter of natural root grafts in *Q. ellipsoidalis* E.J. Hill, the rate of transmission being influenced by host density, soil depth, soil texture, and the occurrence of other species (Juzwik et al. 2011).

Dispersal by water was reported by Vigouroux and Stojadinovic (1990) who established that wounded roots of *Platanus orientalis* L. were infected by *C. platani* spores carried in irrigation water. Dispersal by wind or water occurs more often in the asexual stage because conidia have rounded surfaces, allowing easy removal from the conidiophores. Conversely in the sexual stage, ascospores are produced on perithecia and held together by a sticky, hydrophobic matrix; their concave surfaces also promote adherence to each other (Malloch & Blackwell 1993). The sticky matrix facilitates dispersal by insects or other vectors though reduces transmission by wind and rain, at least when the intensity of wind and rain are low (Harrington 2007).

Wounds created by insects, animal, or human activities through the use of pruning implements or tapping knives, are the most common infection courts for *Ceratocystis* spp. (Hinds 1972; Kile 1993; Teviotdale & Harper 1996; Harrington 2013; Brawner et al. 2015). For example, monkeys strip bark from *A. mangium* to feed on the sweet-tasting cambium and

outer wood of young trees (Hardie et al. 2017). The wounds created act as the initial entry point for the fungal spores into the plant tissues. Direct transmission occurs via known associations with fungal feeding insects (Kile 1993; Harrington 2013), particularly nitidulid beetles and flies (Diptera) (Heath et al. 2009b), which then play an important role as disease vectors (Harrington 2007). These insects feed on mycelial mats in the infected plants which contain abundant perithecia where ascospores are produced (Harrington 2007).

The relationship between fungal feeding insects and *Ceratocystis* is facilitated by the production of fruity odours by *Ceratocystis* and a fermentation exudate (Lanza et al. 1976; Kile 1993; Harrington 2007; Brawner et al. 2015). The fruity aromas contain fatty acids and esters that are toxic to insects which do not rely on fungi for their nutrition. Only insects with high tolerance of mycotoxins, like nitidulid beetles, are attracted to and feed on the mycelial mats of *Ceratocystis* spp. (Kile 1993; Dowd 1995). When the insects feed on the fungi, the sticky ascospores adhere to their bodies; these spores are then transmitted to healthy trees with fresh wounds (Heath et al. 2009a). However, even though the relationship between nitidulid beetles (Coleoptera) or flies (Diptera) and mycelium mats is well recognized, most species of *Ceratocystis* do not have specific insect vectors for their dispersal (Kile 1993).

Although disease transmission in *Acacia* plantations can be caused by all or a combination of the above mechanisms, the initial development of the disease in Indonesia was linked to singling and removal of lower branches to facilitate silvicultural operations (Anthony Francis; pers. comm.). *Acacia mangium* trees were singled in plantations at age 4 – 8 months to reduce the incidence of multiple stems and to increase tree diameter. The singling process creates longitudinal wound of approximately 4-6 cm diameter on the remaining stem. An investigation showed that these practices increased *Ceratocystis* transmission and significantly increased rates of tree death (Tarigan et al. 2010).

II.2. Strategies for ceratocystis disease management

II.2.1. Early detection, surveying and monitoring

Strategies for managing forest diseases and their vectors parallel those used in agriculture: (i) avoidance, (ii) exclusion, (iii) eradication, (iv) protection, (v) host resistance, (vi) curative treatments, and (vii) integrated management (Edmonds 2013; Harrington 2013). However, as plantation forests are usually grown in monoculture over larger areas for several years before harvest and attain a much greater biomass than agricultural crops, the range of viable strategies is often more limited. For example, for environmental and economic reasons, control using pesticides is rarely used because forest estates can be very large in area (Wingfield et al. 2001a; Harrington 2013). As is the case for acacias in S-E Asia the species planted is often an exotic, and *Ceratocystis* has emerged as a new disease. As a result, disease management strategies must be environmentally friendly and based on a clear understanding of the biology of the host and the behaviour of the pathogen (Wingfield et al. 2001a).

In order to respond to pests and diseases, they must first be detected. Diseases can be detected from an on-ground examination of individual trees (Bechtold & Patterson 2005; Carnegie et al. 2018), but modern technologies also allow detection at the landscape level through aerial photography, satellite imagery and LiDAR (light detection and ranging) (Hussin & Bijker 2000; Andersen 2009; Stone & Mohammed 2017). Such landscape-level detection can be followed up by ground-based surveying and monitoring. Together these enable a prediction and a description of changing pest or disease conditions during a growing cycle (Edmonds 2013). Early detection is a preferred strategy as this can be used to prevent increases in disease incidence and the outbreak of epidemics.

II.2.2.1. Ground surveying and monitoring

Surveying and monitoring are used to recognize changes in pest and disease condition. A survey requires a single recorded set of measurements; monitoring involves repeated standardized surveys to ensure that formulated management standards are being maintained (Alexander 2008). As such, monitoring is an essential and integral component of forest planning that can be used to develop management strategies (Ferretti 1997). There are three basic requirements for successful ground monitoring: sampling design, collection of specimens when necessary, and measurements using a variety of different techniques (Ferretti 1997). The sampling design should representative of a larger area and population. This then can be supported with further analysis and observation in the laboratory using the collected specimens. Both sampling design and specimen collection should be conducted using methods that achieve high efficiency and reliability (Bechtold & Patterson 2005).

Ground monitoring in large forest plantations can be inefficient, costly to apply, and time consuming; there is also an inevitable delay in obtaining reliable information that can guide a response to a pest or disease outbreak (Souza et al. 2015). Nevertheless, ground monitoring is still used to support early detection. If combined with landscape-level aerial imagery or more traditional aerial sketch-map surveys, the information can be entered into a geographic information system (GIS) which is used to map the changes in pest and disease incidence and to implement and coordinate control options (Juzwik et al. 2011).

Ground monitoring of ceratocystis wilt disease in forest plantations is an important first step in developing strategies for its control. Records of tree health status, observations of insect vector populations, humidity and other information related to implementing silvicultural practises such as singling and pruning which may affect tree health provide the basis for decision making. These data can improve recognition of changes in trees condition and can be used to trigger implementation of strategies for disease management (Ferretti 1997). Field

sampling and fungal isolation in the laboratory can be used to confirm the identity of the pathogen (Juzwik et al. 2011). However, for *Ceratocystis*, it is also necessary to monitor the presence of nitidulid and ambrosia beetles because of their important role as vectors in spore dispersal (Juzwik 2007; Heath et al. 2009a); there can be a significant correlation between vector activity and disease severity (Hayslett et al. 2008). Climate has also been shown to correlate with insect-vector activity. In South Africa, nitidulid and ambrosia beetle activity is much higher during spring and early summer than in winter months (Heath et al. 2009a). Activities, such as pruning, that lead to wounds should therefore be avoided during periods of high beetle activity (Hayslett et al. 2008; Heath et al. 2009a). There is little information that relates *Ceratocystis* vector activity to tropical climates, though nitidulid and ambrosia beetles are known as important vectors of *C. manginecans* in these conditions (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). Insect activity has been reported to occur around the year (Wolda 1988), though populations tend to be higher during the dry than wet season which is associated with an increased effect of parasitism and plant investment in anti-herbivore defences such as alkaloids and polyphenol (Dyer et al. 2012). Thus, the monitoring of the population and activity of these insects in relation to wounding may also assist disease control of *Ceratocystis*.

II.2.2.2. Remote sensing and Geographic Information Systems (GIS)

Remote sensing collects data by detecting the electromagnetic energy that is reflected from the earth's surface (Pidwirny 2006). A geographic information system (GIS) is a dynamic computer-based tool for mapping and analysing features on the earth's surface that uses space-time coordinate values (Sakai 2012). Both remote sensing and GIS have been used in forestry and agriculture to develop resource inventories, and for harvest planning, fire management, map production, and ecosystem management (Sonti 2015).

Remote sensing of forest pests and diseases has been applied since aerial photography first became available in the 1930's (Coppin & Bauer 1996). Combined with GIS, this approach is used to complement ground-based surveys because of its ability to extend spatial coverage, as well as offering objective assessments of forest health (Wulder et al. 2006). For example, in China, by setting the spectral wavelength range to 350 –1000 nm, hyperspectral remote sensing combined with measurements of chlorophyll content were successfully used to detect pine wilt disease of *Pinus massoniana* caused by the pine wood nematode, *Bursaphelenchus xylophilus*; the chlorophyll content of healthy trees was detected between 650-680 nm and infected trees at <600 nm because of the reduction of chlorophyll content (Ju et al. 2014). In western North America, high resolution airborne multispectral imagery using a Compact Airborne Spectrographic Imager (CASI) was used to detect root-rot disease symptoms caused by *Phellinus weirii* from assessments of subtle changes in crown shape, changes in growth rate, needle loss and mortality; these visual symptoms were delineated to the spectral imagery (Leckie et al. 1998). Even though chlorotic symptoms were difficult to detect consistently, CASI was able to distinguish moderate to severe levels of damage, and tree death.

Remote sensing by using Colour-infrared (CIR) digital imagery was used by Everitt et al. (1999) to detect oak wilt disease caused by *B. fagacearum.* The imagery was first interpreted from the radiometric reflectance measurements and then verified by ground observations. The data was then linked to leaf chlorophyll concentration using three visible spectral bands: visible green (520 to 600 nm), visible red (630 to 690 nm) and near-infrared (760 to 900 nm) that was used to distinguish between healthy, infected and dead oak trees; infected trees had veinal necrosis and tip burn (Everitt et al. 1999).

A camera mounted in an Unmanned Aerial Vehicle (UAV) was used to detect wilt disease caused by *C. fimbriata* on *Eucalyptus* spp. in Brazilian plantations (Souza et al. 2015). The images were analysed and compared using four distinct machine learning techniques: K-

Nearest Neighbours (K-NN) which classify a test sample according to a distance metric; Random Forest (RF) which uses a combination of tree predictors where each tree depends on the values of a random vector sampled independently and with the same distribution for all trees in the plantation; Artificial Neural Network (ANN) which is a biologically inspired method based on the function of the human brain and non-linear mapping structures; and Gaussian Processes (GP) which is a Bayesian nonparametric tool that learns the input-output transformation function based on training data. The GP learning model was best able to reliably and accurately distinguish between healthy and infected trees, and was suitable for detecting wilt disease in other species and other diseases affecting large-scale plantations. A spectral signature of *Ceratocystis*-infected *Metrosideros polymorpha* has been detected at leaf and canopy level and provides a basis for mapping and monitoring of disease spread in Hawaii (Asner et al. 2018). A clear timeline of symptom development is the first requirement for aerial assessment of ceratocystis disease in acacias.

II.2.2. General approaches for managing vascular diseases of woody plants

Most approaches for managing vascular diseases are based on plant species, location, total planting area and silvicultural practice. For *Ceratocystis*, the most frequently suggested management strategy for preventing infection is wound avoidance (Kile 1993; Harrington 2013). The role of wounds in disease epidemics is well-established. For example, the incidence of vascular wilt disease (true wilt) in *Quercus virginiana* and *Q. fusiformis* in Minnesota increased if pruning was undertaken in the spring and early summer as nitidulid beetles are very active and populations significantly increase with the rapidly warming temperatures (Hayslett et al. 2008), though pruning in wet seasons can also be conducive to disease spread (Haugen et al. 2008). In South Africa, infection by *Ceratocystis albifundus* and

tree death in *A. mearnsii* plantations occurred after the branches were pruned for fuel wood (Roux & Wingfield 2009). Rapid development of wilt and canker symptoms and mortality in *A. mangium* plantations in Indonesia caused by *Ceratocystis manginecans* occurred after 6-to-8-month-old trees were pruned or singled, leaving a longitudinal wound of 4-6 cm on the remaining stem (Tarigan et al. 2010). Bark stripping by monkeys and squirrels also leaves large wounds on acacia stems and branches in Indonesian plantations, as can elephant damage and wind (pers. obs.), all of which can create suitable entry points for *Ceratocystis* infection.

Removal of symptomatic trees and stumps as well as neighbouring trees that may have been infected by *Ceratocystis* may help to limit disease spread (Kile 1993). This approach aims to minimize the inoculum load. The material is then removed from the plantation in order to avoid fungal dispersal into healthy trees via insect vectors, root grafts, wind and rain (Kile 1993; Harrington 2013).

Fungicides applied by stem injection into xylem tissue can be used to control sap stain and canker disease caused by *Ceratocystis* and similar pathogen which colonise the vascular system, such as *B. fagacearum*. Injecting 20 ml of 14.3% propiconazole into the root collar of young *Quercus rubra* (red oak) two weeks before inoculation by *B. fagacearum* delayed disease development and extended the life of infected trees for at least two years (Blaedow 2009). Two years after injection, the fungicide could still be detected at the point of injection as well as in the primary roots and lower stem, showing that propiconazole can move through xylem tissues and remain stable in the tree for an extended period. However, its concentration decreased with distance from the injection point which would have reduced its ability to neuter the infection (Blaedow 2009). Despite the potential effectiveness of some fungicides in protecting trees from ceratocystis disease, this can be a high-cost management strategy which is not feasible in forest plantations. This technique may only be useful for protecting high-value trees such as

gardens or seed orchards. Additionally, it may only delay the expression of disease symptoms, and some of these chemicals are toxic to other organisms.

Wound dressings can provide a physical barrier to *Ceratocystis* infection as well as inhibiting fungal growth (Harrington 2013) and low toxicity treatments have been developed. Application of latex paint to pruning wounds in *Quercus* spp. has been shown to be non-toxic and effectively reduced wilt disease (French & Juzwik 1999; Camilli et al. 2007).

Because of the limitations of silvicultural and chemical approaches to disease control, the selection of resistant host materials is currently considered the most effective and economic strategy for managing ceratocystis disease (Zauza et al. 2004; Harrington 2013). Such an approach requires genetic variation within host species that has the potential to be exploited to increase the resistance of trees to disease through breeding. Trees with higher levels of genetic resistance may then protect themselves from fungal infection. A successful example is *Platanus acerifolia* (London plane); trees resistant to *Ceratocystis platani* were obtained from breeding and screening processes that remain free of canker and sap stain symptoms (Harrington 2013).

Genetic resistance refers to the heritable features of a host genotype that suppress biotic interference, or features that result in higher productivity and quality compared with other genotypes at the same initial level of biotic infestation and under similar environmental conditions (Kennedy & Barbour 1992). Genetic resistance to disease in plants can be monogenic and controlled by a single major gene which is race-specific and relatively easy to identify and manipulate in a breeding program; it can be expected to provide complete resistance (Poland et al. 2009). However, such resistance only provides protection against a specific strain or race of the pathogen; within a short time, it may easily be broken down (Maramorosch & Loebenstein 2009). Genetic resistance can also be polygenic and controlled by a number of genes (Brun et al. 2010), and this provides a similar level of protection against all the races of a pathogen. Although it may be difficult to identify and manipulate the genes

in a breeding program to confer this type of resistance, if successful it is not easily broken down by the pathogen and can be expected to give the host long-lasting protection (Lindhout 2002). Breeding for polygenic resistance is better suited to acacia forestry which uses clonal stock or improved seedlings from a range of single families.

So in forestry, breeders work with population genotypes of both host and pathogen in order to protect a range of genotypes of a given species from all known races of a pathogen (Carson & Carson 1989). As trees, unlike annual crops, are long-lived, and plantation growing cycles are usually several years, such genetic polygenic resistance against disease is expected to have a long-term utility in trees (Sniezko 2006). The development of resistant clones may be expedited by mapping quantitative trait loci (QTLs), as has been done for *Ceratocytis* in eucalypts in Brazil (Rosado et al. 2016).

II.2.3. Options for control of wilt diseases in forest and/or plantation trees

To date, efforts to manage ceratocystis canker and wilt disease in planted *Acacia* trees have focused on avoidance by better managing the causes of wounding, minimizing wound size, limiting pruning and singling activities to periods when insect vectors are less active, and screening and breeding for clonal resistance (Heath et al. 2009a; Brawner et al. 2015). Tarigan et al. (2011b) applied two pruning treatments to *A. mangium* and *A. crassicarpa*. The wounds were then inoculated with a *Ceratocystis* fungal isolate. In one treatment, branches were carefully removed with secateurs just outside the branch collar, taking care not tear the bark; in the other treatment, during branch removal, the branch collar and bark below were torn, creating a flap. In the first and second treatments, average lesion lengths were 17 mm and 450 mm, and 11 mm and 406 mm in *A. mangium* and *A. crassicarpa*, respectively. Careful pruning that created small wounds resulted in lower disease severity than larger, ragged wounds.

Where wounding is unavoidable, wound dressings to protect the trees from *Ceratocystis* infection are recommended (Harrington 2013).

As for other diseases, the selection and breeding of disease tolerant materials is considered the most cost effective approach for dealing with *Ceratocystis* (Roux & Wingfield 2009), though to date no resistant clones have been identified. In clonal systems, the planting of a diversity of planting stock rather than single clones should be practised (Wingfield et al. 2001a). In a planting in Brazil of 18 commercial clones of *Eucalyptus grandis* × *E. urophylla* hybrid, four clones expressed no discoloration symptoms on the stem and showed an ability to overcome fungal infection caused by *C. fimbriata* (Zauza et al. 2004). When five elite genotypes of *E. grandis* and 16 of *E. urophylla* were inoculated with *C. fimbriata*, four and eight of the respective genotypes of *E. urophylla* were tolerant and able to reduce the lesion length in the progeny population up to 74.4% (Rosado et al., 2010).

Biological control is considered a cost effective and environmentally friendly management tool for pests and diseases in forest plantations, though there are very few reports of their successful development for vascular wilt and canker diseases (Wingfield et al. 2001a; Garnas et al. 2012). Endophytic microorganisms may be one option for woody trees. Four fluorescent pseudomonads, *Pseudomonas fluorescens* Migula strain WCS374r, *P. fluorescens* WCS417r, *P. putida* (Trevisan) Migula strain WCS358r and *P. aeruginosa* 7NSK2 were introduced into the roots of *E. urophylla* seedlings growing in soil infested with the bacterial wilt pathogen *Rasltonia solanacearum*; *P. fluorescens* WCS417r suppressed disease incidence by 45% and was able to induce systemic resistant (ISR) against *R. solanacearum* (Ran et al. 2005).

II.2.3.1. Resistant and tolerant trees

Plants generally have defence mechanisms that enhance their ability to survive negative impacts from biotic or abiotic stress (Vale et al. 2001). The first line of defence to pest and disease invasion is avoidance which includes physical barriers such as bark, hairs, thorns and resin ducts. Avoidance generally works as a defence mechanism against animal attack (Vale et al. 2001). If avoidance is ineffective, resistance and tolerance mechanisms come into play after contact has been established and these mechanisms are more common for protecting plants from infection by fungal pathogens (Vale et al. 2001).

Schafer (1971) has defined tolerance as the ability of a cultivar to minimize loss of yield or quality, in comparison with other cultivars, after pathogen infection, whereas resistance is the ability of a plant to overcome the infection process and prevent development of the pathogen, resulting in a disease-free plant. Monogenic and polygenic resistance are considered more important for plant defence than avoidance and tolerance (Vale et al. 2001), and the best way to deal with plant disease problems in forest trees (Wingfield et al. 2001a; Harrington 2013).

II.2.3.1.1. Selection and breeding for disease resistance

The process of obtaining planting material incorporating disease resistance is generally started by screening to identify putative resistant trees and then continued with breeding techniques leading to the propagation of resistant plants (Wingfield et al. 2001a). Individuals possessing disease resistance (or tolerance) genes may be propagated in conventional systems or by *in-vitro* tissue culture (Wenzel 1985).

Screening for resistant or tolerant plants has been recognized as a major component of breeding programs. The plants which will be screened, as young as possible, are exposed to high levels of pathogen inoculum and those with the lowest levels of disease severity selected

(Vale et al. 2001). This is potentially a slow process because of the large numbers of plants that must be screened, the need to understand what levels of pathogen inoculum are required and the appropriate conditions for infection, and the right stage of growth to infect the host. If all this information is known, the screening of acacia trees for resistance or tolerance to *Ceratocystis* should be possible in about six months (Wingfield et al. 2001a; Van Wyk et al. 2010; Brawner et al. 2015). Shorter periods should be possible if leaf rather than stem inoculation methods (Magalhães et al. 2016) can be developed for acacias.

Genetic modification offers an alternative approach. This usually starts with mapping plant genomes with molecular markers and placing resistance genes on the map by linkage analysis (Wingfield et al. 2001a). Genes are then transferred between plant species, from microorganisms to plants, or by preparing new genetic constructs from combinations of isolated genes through a transgenic process (Vale et al. 2001). Transfer of an *Arabidopsis thaliana cysteine proteinase* inhibitor (*Atcys*) gene into *Populus alba* was mediated by *Agrobacterium tumefaciens* in tissue culture resulting in trees resistant to *Chrysomela populi* (poplar leaf beetle); the digestive proteinase activity of the beetle larvae was inhibited (Delledonne et al. 2001). Similarly, synthetic antimicrobial peptide ESF39A transferred into *Ulmus americana* using a similar procedure reduced disease levels of *Ophiostoma novo-ulmi* (= *Ceratocystis ulmi*) (Dutch-elm disease) on *Ulmus Americana*; potential host resistance to this disease was also enhanced (Newhouse et al. 2007).

II.2.3.1.2. Current screening protocol against vascular wilt disease in forestry

Breeding for resistance in trees against plant pathogens involves the selection of a range of phenotypes in natural forests or forest plantations and then conducting pathogenicity tests either *in situ* or, more commonly, by using artificial inoculation under controlled conditions (Sniezko 2006; Tarigan et al. 2010). This enables selection of putative genes that may confer disease resistance before the breeding program commences. This is currently considered the

most effective method for controlling ceratocystis vascular wilt and canker diseases in forest plantations (Wingfield et al. 2001a; Rosado et al. 2010; Harrington 2013). The trees with the highest rates of survival and lowest levels of disease symptoms are selected for inclusion in breeding programs (Sniezko 2006).

Inoculation tests *in situ* (natural) can provide the best information for a given location because the influence of the natural environment on host-pathogen interactions is captured (Brawner et al. 2015). In addition, seedlings may not respond in exactly the same manner as older trees (Van Wyk et al. 2010), however, there is inevitably a risk of disease spread from the introduced pathogen if inoculations are carried out *in situ*. This approach also first requires trees rather than seedlings, which slows the screening process (Tarigan et al. 2010; Sniezko & Koch 2017).

Artificial inoculations using fungal isolates are conducted by wounding tree stems or branches before placing a plug of mycelium or spore suspension of the pathogen under the bark. This is normally carried out on juvenile plant material under controlled conditions (Green et al. 1985; Tarigan et al. 2010). Levels of resistance to ceratocystis wilt and canker disease are evaluated by measuring the length of xylem discoloration (Roux et al. 1999; Zauza et al. 2004). This screening method is time-consuming as disease resistance is only expected to occur in a low percentage of the population. As large numbers of cultivars or clones must be used, rapid screening technologies are required (Brawner et al. 2015). One possible option for *Ceratocystis* is leaf inoculation that has been shown to give a similar result to tree wounding in screening assays using leaves of *Castanea dentata* (American chestnut), *C. molissima* (Chinese chestnut) and *C. pumila* (Allegheny chinquapin or dwarf chestnut) against *Cryphonectria parasitica* (blight fungus) (Newhouse et al. 2014). Leaf assays also reflected the level of resistance in cacao plants against *Ceratocystis* (Magalhães et al. 2016).

II.2.3.2. Biological control

Biological control encompasses the use of fungi, bacteria, actinomycetes and viruses to suppress or decrease the population density of a plant pathogen (Bale et al. 2008). These microorganisms or biological control agents (BCAs) may also have beneficial interactions internally or externally with their host plants (McInroy & Kloepper 1994). The attraction of biological control is that it can be applied to broad or narrow targets depending on the biocontrol organism, is less site-specific and less prone to build-up of resistance, and is cost-effective for specialized applications; as chemical use is reduced or eliminated, environmentally it can be a preferred option; it can also be integrated with other control strategies, and may enhance plant growth (Whipps & Lumsden 2001).

In practice, there are few examples of successful biological control in forest trees (Wingfield et al. 2001a). This may be related to their larger biomass, more complex anatomy and greater longevity than agricultural and horticultural crops. The inoculum load of the pathogen also has an opportunity to build up during the rotation, creating greater challenges if the BCA is to be successful (Cazorla & Mercado-Blanco 2016). For example, repeated infection of the forest through a large root system by a soil borne pathogen allows the disease to persist in soils as dormant, quiescent or resistant propagules such as chlamydospores and microsclerotia after tree death and after the trees are harvested (Cazorla & Mercado-Blanco 2016). These propagules can then infect new trees or any root tissues that are not reached by the BCA, thereby reducing its effectiveness.

A success story in forestry has been use of the fungus *Phlebiopsis gigantea* for controlling root-rot disease caused by *Heterobasidion annosum* in pine trees. This fungal pathogen can colonize and survive in the stumps of harvested trees which then act as an inoculum source. Application of *P. gigantea* spores to the surface of freshly cut stumps results in rapid colonization of the wood and inhibits the growth of *H. annosum*, reducing disease incidence in

the next rotation (Rishbeth 1979). First produced in 1961, this commercially-produced BCA is used routinely to control root-rot disease in pine plantations in the northern hemisphere (Pratt et al. 2000). While a potential *Phlebiopsis* sp. has been identified that shows a mycoparasitic reaction against *Ganoderma philippi*, a root rot pathogen that kills *A. mangium* (Agustini et al. 2014), further refinement of application methods is required to commercialise this fungus as a BCA.

Do BCAs have any potential application in controlling vascular wilt diseases? Injection of 100 to 400 ml (10^8 cells/ml) of *Pseudomonas syringae* using a specially developed 'gouge-pistol' into base of trunks of *Ulmus* sp. suppressed Dutch Elm disease caused by *Ophiostoma ulmi* and resulted in a high proportion of healthy trees (Scheffer 1983). However, due to a lack of correlation between *in vitro* and field tests, it was concluded that *P. syringae* had triggered plant defence against *Ophiostoma* infection through induced resistance rather than antagonism (Scheffer 1990; Scheffer et al. 2008). Another potential BCA, *Verticillium dahliae* isolate WCS850 was injected into mature clonal trees of *Ulmus carpinifolia* that were either resistant or susceptible to *C. ulmi*; both types of trees were able to suppress disease infection (Scheffer 1990). Injecting *V. dahliae* isolate Vd-48 into 6-7 years old *Ulmus minor* reduced wilting symptoms associated with *Ceratocystis* infection (Solla & Gil 2003). Both studies reported that *V. dahliae* could be re-isolated from at or near the injecting point, an indication that translocation of this fungus had been minimal and any contact with *C. ulmi* unlikely. Thus *V. dahliae* most likely acts by triggering host defences against *Ceratocystis* infection.

II.2.3.2.1. Endophytic bacteria

Bacteria that live internally in plant tissue without causing any negative impact on their host are recognized as endophytic bacteria (Schulz & Boyle 2006). Their presence in roots, stems, branches, leaves, fruits, and xylem sap was discovered more than 120 years ago (Hardoim et al. 2008). A mutualistic relationship between some of these bacteria and their host

plants was recognized in 1926, along with their ability to live as obligate or facultative endophytes at different stages in their life cycle (Hardoim et al. 2008). Obligate endophytes are strictly dependent on their host plants and transmitted to other plants through vectors; facultative endophytes live inside their host plants for part of their life cycle, but can also exist independently at other stages of their cycle (Hardoim et al. 2008).

Endophytic bacteria express symbiotic, mutualistic and commensal relationships with their host plants (Ryan et al. 2008), and their potential for controlling plant diseases in crops has been explored since Hollis, in 1951, discovered endophytic bacteria in potato plants (Lodewyckx et al. 2002; Izumi 2011). As well as protection from pests, diseases and weeds, these relationships may also provide other benefits to the host: enhancement of growth, adaptation to environmental stress and tolerance to heavy metal contamination (phytoremediation) (Lodewyckx et al. 2002; Bacon & Hinton 2006).

The majority of research into endophytic bacteria has been with agricultural or horticultural crops (Kobayashi & Palumbo 2000). In agricultural crops, endophytic bacteria have been introduced into *Gossypium hirsutum* L. (cotton) for controlling vascular wilt disease caused by *Fusarium oxysporum* Schltdl. (Chen et al. 1995). Southern blight disease caused by *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr. which infects the medicinal plant *Atractylodes lancea* (Thunb.) DC can be reduced by applying another endophytic bacterium, *Pseudomonas fluorescens* Migula which produces the lytic enzymes xylanase and pectinase, the antibiotic 2,4-DAPG and siderophores which together inhibit as well as kill *A. rolfsii*; disease incidence was reduced by 70% (Zhou et al. 2014).

Nevertheless, several species of endophytic bacteria have been isolated from woody species: *Bacillus alvei* Cheshire & Cheyne (syn. *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al.) and *B. megaterium* de Bary from *Quercus serrata* Murray (oak), *Arthrobacter* spp. and *Agrobacterium* spp. from *Pinus sylvestris* L. (Scots pine), *Bacillus subtilis* (Ehrenberg) Cohn,

B. pumilus Meyer & Gottheil, *B. sphaericus* Neide, *Paenibacillus macerans* Schardinger and *Serratia marcescens* Bizio from *Pinus taeda* L. (Loblolly pine), *Pseudomonas fluorescens* Migula and *Arthobacter citreus* Sacks from *Picea mariana* (Mill.) Britton, Sterns & Poggenburg (Black spruce), *Pseudomonas* spp. and *Arthrobacter* spp. from *Picea glauca* (Moench) Voss (white spruce), *Erwinia salicis* (Day) Chester from *Populus alba* L. (aspen), and *Bacillus* spp., *Paenibacillus* spp., *Sphingomonas* spp., and *Burkholderia* spp. from *Eucalyptus* spp. (Kobayashi & Palumbo 2000; Bacon & Hinton 2006; Izumi 2011). The large biomass and perennial nature of these hosts provide a stable habitat for this wide diversity of endophytes (Izumi 2011), and the possibility that they could play a role as BCAs against vascular diseases such as *Ceratocystis* (Yadeta & Thomma 2013).

II.2.3.2.2. Role of endophytic bacteria in plants

Endophytic bacteria play an important role in the growth of their hosts, both directly and indirectly by protecting the plant from infection by disease-causing organisms (Kobayashi & Palumbo 2000). However, there is very limited information on their role in disease management in forest trees (Chanway 1998).

Endophytic bacteria can enhance plant growth by assisting nutrient acquisition through fixing atmospheric dinitrogen (N_2) into forms that can be taken up by plants. This process is supported by diazotrophic endophytes such as *Herbaspirillum* sp., *Azorhizobium* sp., *Azoarcus* sp., *Azospirillum* sp., *Acetobacter* sp., and *Pseudomonas fluorescens*, and endosymbiotic *Rhizobium* species that reside in root nodules (Carvalho et al. 2014). As diazotrophic endophytes are sequestered within the plant, the fixed forms of nitrogen, NH_4^+ or NO_3^- are available for rapid uptake (Bacon & Hinton 2006). Endophytic bacteria can also enhance the acquisition of nutrients from the soil by converting insoluble minerals into soluble forms (Wakelin et al. 2004; Bacon & Hinton 2006). They also can produce phytohormones such as auxins, gibberellins or cytokinins which can increase plant growth (Bacon & Hinton 2006).

In adverse environments that lead to plant stress, endophytic bacteria can produce the enzyme *1-aminocyclopropne-1-carboxylic acid* (ACC) *deaminase* which encourages the plants to reduce the production of the hormone ethylene. Ethylene is an important modulator of plant growth and features in plant responses to a wide range of stress factors (Abeles et al. 2012). It can also help plants to activate defence mechanisms through its function as a key signalling molecule (Clarke et al. 2000). When plants are stressed, they produce high levels of ethylene which can inhibit indole acetic acid (IAA) signal transduction; as IAA stimulates plant cell-wall permeability, the efficient use of water may be threatened. Thus, bacterial ACC may assist the plant to decrease ethylene production and stimulate cell-wall permeability, thereby assisting plant responses to drought and osmotic stress. and soil salinity (Cheng et al. 2007; Glick et al. 2007). *Achromobacter piechaudii* ARV8, a plant growth promoting bacterium which was isolated from the Arava region of southern Israel, has the ability to produce ACC deaminase (Mayak et al. (2004). This bacterium in 40 ml suspension was poured into tomato seedlings growing in a high salt concentration (up to 172 mM NaCl) and resulted in a significant increase in the fresh and dry mass of tomato seedlings. In addition, ethylene production was reduced and uptake of phosphorus and potassium by the seedlings increased, suggesting some alleviation of salt concentration. Thus, the bacterium may not only assist seedlings to alleviate the effect of salt but also increase seedling growth in saline environments.

Endophytic bacteria can also enhance the ability of plants to grow in environments contaminated by chemicals or heavy metals (phytoremediation) (Ryan et al. 2008). Peas (*Pisum sativum*) were inoculated with *Pseudomonas putida* strain POHV6, an endophyte that was isolated from poplar trees (*Populus trichocarpa x deltoids* cv. Hoogvorst) grown in soil contaminated with 2,4-D. The bacterium actively colonized the rhizosphere and internal plant tissues, allowed the plants to maintain normal rates of growth, and increased uptake of 2,4 D into the plant tissues where it was quickly degraded (Germaine et al. 2006). In this context, an

endophytic bacterium protected the plants from the toxic effects of high levels of a herbicide and reduced its concentration in the contaminated soil.

II.2.3.2.3. Location and mechanisms of translocation in plant tissue

Bacterial endophytes invade their host plants passively through wounds, and natural openings such as lenticels, stomata, root hairs and natural cracks in the lateral roots (Sprent & De Faria 1988; Hallmann et al. 1997). This process is similar to a bacterial pathogen entering the plant tissue (Kobayashi & Palumbo 2000).

Once in the plant tissue, endophytic bacteria quickly grow and multiply, colonizing the inter- and intra-cellular spaces. They may then be localized in a specific tissue such as the root cortex, or transported through conducting elements or the apoplast (Hallmann et al. 1997). Bacterial endophytes in agricultural crops are most abundant in the roots (10^5cfu/g) and densities then decrease gradually, 10^4cfu/g in stems and 10^3 cfu/g in leaves (Quadt-Hallmann & Kloepper 1996). The plant tissues provide a comfortable environment for endophytes, protecting them from extremes of temperature, low osmotic potential, ultraviolet radiation and interspecific competition (Hallmann et al. 1997).

The speed of movement of endophytes within plants varies with the tissue and strain of endophyte, moving more slowly in stem than root tissue (Hallmann et al. 1997). After introducing *Aureobacterium saperdae* and *Phyllobacterium rubiacearum* into cotton, both bacteria were detected 3-5 cm from the inoculation point 14 days after inoculation; there was no additional movement 20 days inoculation (Chen et al. 1995). In *Pyrus communis* L. inoculated with the bacterial endophyte *Pseudomonas syringae*, movement was detected 3 cm from the inoculation point in the stem tissue after 20 days but there was no movement upwards following root inoculation due to limited vascular mobility (Whitesides & Spotts 1991). This occurs because the root endodermis regulates flow to the vascular tissues, limiting the movement of solutes and endophytic bacteria from the root cortex (Hallmann et al. 1997).

Endophytic bacteria, however, may move into vascular tissues when the endodermis is broken during secondary root formation as this creates a conducting element or an apoplastic pathway to make this possible (Peterson et al. 1981). Once in the vessels, the bacteria can be transported acropetally through systemic movement to all plant tissues. Three strains of *Pseudomonas putida* introduced into the roots of *Populus trichocarpa x deltoides* var. Hoogvorst were detected using the green fluorescent protein (gfp): kanamycin cassette. Ten weeks after inoculation, all three strains of bacteria had colonized all the root tissues and two strains were found in stems and leaves, confirming the systemic movement of these bacteria (Germaine et al. 2004).

II.2.3.2.4. Potential mechanisms of control against plant pathogen

Bacterial endophytes suppress plant diseases through the production of enzymes, antifungal and antibacterial compounds (allellochemicals), by competition with pathogens for nutrients or niches and stimulation of induced systemic resistance (ISR) (Compant et al. 2005; Bacon & Hinton 2006). Thus endophytic bacteria exert control of the pathogen through antibiosis by producing antibiotics or toxins, lysis of its cell wall by excreting chitinase, cellulase and glucanase, and direct competition through antagonism (Berg & Hallmann 2006). In this way, endophytic bacteria act as biological control agents (BCAs) in the control of plant diseases (Kloepper & Ryu 2006). Endophytic bacteria may also overcome plant pathogens by indirect contact; plant resistance is triggered by ISR (Kloepper & Ryu 2006).

All plants respond to infection by pathogenic and non-pathogenic microorganisms through defence mechanisms (Kloepper & Ryu 2006; Choudhary et al. 2007). If elicited by a pathogenic microorganism this is known as systemic acquired resistance (SAR) which usually provides long-term resistance to subsequent pathogen attack and involves the accumulation of pathogenesis-related (PR) proteins (chitinase and glucanases) and salicylic acid (SA) which act as an endogenous signal compound for pathogen recognition and export of the signal for

inducing a defence response by plant (Mauch-Mani & Métraux 1998; Choudhary et al. 2007). Systemic defence associated with endophytic bacteria is further stimulated by the plant hormones jasmonic acid (JA) and ethylene (ET) (Choudhary et al. 2007; Shoresh et al. 2010). The plants which have been infected by endophytic bacteria can respond through increased production of JA and ET as signalling regulators. These plant hormones then spread systematically through the plant, induce subtle changes in gene expression in yet uninfected plant parts, and finally to systemic expression of broad spectrum and long-lasting disease resistance (Heil & Bostock 2002; Choudhary et al. 2007).

Several studies have reported endophytic bacteria acting as potential antagonistic BCAs and their role in induced systemic resistance. For instance, Melnick et al. (2011) isolated and identified sixty-nine endophytic bacterial isolates from leaves, pods, branches and flowers of *Theobroma cacao*. These bacteria were then tested *in vitro* against *Moniliophora* spp., the fungus which causes frosty pod disease on cacao. Sixteen isolates of endophytic bacteria inhibited the growth of *Moniliophora* spp. through chitinase production, indicating their potential as BCAs. Foliar application of *Bacillus cereus* BT8 to cacao seedlings significantly reduced leaf infection by *Phytophthora capsici* in detached leaf assays (Melnick et al. 2008). The bacteria were only detected in or on treated leaves and no bacteria were isolated from new leaves that grew after application of *B. cereus* BT8, indicating that the bacteria were not capable of movement in vascular tissues. Endophytic colonization, a lack of systemic movement and subsequent disease suppression indicated that *B. cereus* BT8 induced host systemic resistance. Another endophyte, *Enterobacter cloaca*, produces an antibiotic that is effective against *Fusarium moniliforme* in cotton (Hinton & Bacon 1995).

Wilt disease caused by *Fusarium* sp. in cotton was also reduced significantly after inoculation with endophytic bacteria which had been isolated from the same host. Two strains of endophytic bacteria, *Aureobacterium saperdae* and *Pseudomonas putida*, induced systemic

resistance in their hosts and reduced disease symptoms (Chen et al. 1995). *Bacillus oryzicola* YC7007 (2.0 x 10^7 cfu/ml) applied to rice seedlings by root drenching and leaf spraying significantly reduced bakanae disease caused by *Fusarium fujikuroi* by 46-78% via antibiotic production and ISR by stimulating the plant to produce a hormonal defense mechanism which can inhibit *F. fujikuroi* growth by 46 – 78% (Hossain et al. 2016).

II.2.3.2.5. Application of endophytic bacteria in controlling tree diseases

Studies of the beneficial effects of endophytic bacteria in woody trees have focused on their role in promoting growth (Table II-2). *Bacillus subtilis*, *B. pumilus* and *B. licheniformis* extracted from eucalypt trees and introduced into cuttings of the hybrid *E. urophylla* × *E. grandis* by immersion in 10^3 CFU/ml of bacterial suspension significantly enhanced height and root growth through phosphate solubilization and biological nitrogen fixation (Paz et al. 2012). Of 720 putative endophytic bacteria isolated from *Pinus pinea* and *P. pinaster*, 38% had the capacity to increase the mobilization of nutrients and enhance root growth through stimulating plant hormonal production. The endophytic bacteria producing a siderophore involved in mobilizing phosphate were isolated from *P. pinaster*; those isolated from *P. pinea* produced auxin and jasmonate (Barriuso et al. 2005). Plant growth promoting rhizobacteria (PGPR) also produce enzymes and antibiotics that can prevent or reduce pathogen infection in a similar manner to endophytic bacteria. Thus rhizobia can play an indirect role as biological control agents (Ryan et al. 2008).

There are few reports of endophytes acting as BCAs in forest trees (Chanway 1998). However, there are obstacles or challenges which have led to this delay of application of endophytes in forest management. Assay-based selection of suitable endophytes remains an important challenge as a high correlation between laboratory assays and field results is required. The relationship between an introduced endophyte and others in the environment also needs to

be considered. Thus, a selected endophyte may be replaced by others when deployed, or it might act in a more advantageous way when interacting with other, unselected competing endophytes. Hence the need for improvement of assay-based selection techniques (Newcombe 2011) to ensure that the environment where the introduced endophyte is to be inoculated leads to the expected results (Hilszczańska 2016).

Nevertheless, a potential for using endophytes for controlling tree diseases has been demonstrated. For example, the endophytic bacterium *Bacillus pumilus* JK-SX001 has been shown *in vitro* and in a greenhouse test to produce secondary metabolites which reduced disease incidence of poplar canker disease caused by *Cytospora chrysosperma, Phomopsis macrospora* and *Fusicoccum aesculi* by 76.1%, 75.8% and 65.2%, respectively; this endophyte also promoted growth (Ren et al. 2013). *Bacillus amyloliquefaciens* and *B. stearothermophilus,* used to control charcoal root disease caused by *Macrophomina phaseolina* in *Pinus radiata,* reduced seedling mortality in a greenhouse to 7% through production of a secondary metabolite (Valiente et al. 2008).

The potential of endophytic bacteria in managing vascular diseases in forest trees such as bacterial wilt disease caused by *Ralstonia solanacearum* and *Ceratocystis* wilt disease has been investigated. As both the bacterial endophytes and pathogen live in the vascular system, endophytic bacteria have potential as antagonists through the production of secondary metabolites, antibiosis and competition (Yadeta & Thomma 2013). Application of *Pseudomonas fluorescens* and *P. putida* into the roots of *Eucalyptus urophylla* reduced the incidence of bacterial wilt caused by *R. solanacearum* by 45 % (Ran et al. 2005). Endophytic bacteria have been applied to control oak wilt disease caused by *B. fagacearum.* A total of 889 endophytic bacteria were obtained from healthy oak trees (*Quercus fusiformis*), and 183 isolates of mostly *Bacillus* spp. and *Pseudomonas* spp. showed high levels of chitinase activity that strongly inhibited *B. fagacearum* in an *in vitro* assay. Two isolates, *Pseudomonas*

denitrificans 1-15 and *Pseudomonas putida* 5-48 were selected and injected into potted oak trees which were then inoculated with a spore suspension of *B. fagacearun*. The experiment showed that *P. denitrificans* 1-15 was able to reduce oak wilt disease incidence by 50% and decreased the proportion of crown loss by 17% (Brooks et al. 1994).

Even though endophytic bacteria can play a role as antagonists through antibiosis or the production of secondary metabolites to overcome a forest disease like *Ceratocystis* wilt and canker disease, their slow movement in plant tissues is a potential problem as contact with the pathogen may be limited in an organism as large as a tree (Hallmann et al. 1997). However, the ability of endophytic bacteria to elicit ISR in woody plants suggests this may be a more workable pathway and a useful supplement to breeding resistant and tolerant varieties (Percival 2001).

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Table II-2. Studies of endophytic bacteria in woody plants and their potential as growth promoters or biological controls for tree diseases.

Plant	Endophytic bacteria	Effect on the plants	Modes of action	References
Populus sp.	*Enterobacter* sp. *Stenotrophomonas maltophilia* *Pseudomonas putida* *Serratia proteamaculans*	Increased biomass production and root development.	Produced plant growth promoting compound and phytohormones	Taghavi et al. (2009)
Panax ginseng	*Bacillus megaterium* *Bacillus cereus* *Micrococcus luteus* *Lysinibacillus fusiformis*	Growth promotion	Indole Acetic Acid (IAA) and siderophore production, solubilized mineral phosphate	Vendan et al. (2010)
Pinus pinea *Pinus pinaster*	*Bacillus* sp.	Increased nutrient mobilization and enhanced root growth by 38 %.	Phosphate solubilization and auxin production.	Barriuso et al. (2005)
Eucalyptus urograndis	*Bacillus subtilis* *Bacillus pumilus* *Bacillus licheniformis*	Enhanced growth of roots and aerial length of *Eucalyptus* seedlings	Production of IAA and phosphate solubilization	Paz et al. (2012)
Theobroma cacao	*Bacillus subtilis* *Enterobacter cloacae*	Promoted vegetative growth of cacao seedlings.	Produced growth promoting compounds.	Leite et al. (2013)

Table II-2. Studies of endophytic bacteria in woody plants and their potential as growth promoters or biological controls for tree diseases (continued)

Plant	Endophytic bacteria	Effect on the plants	Modes of action	References
Theobroma cacao	*Bacillus mycoides* *Bacillus pumilus* *Bacillus cereus*	Reduced severity of disease caused by *Phytophthora capsici* in cacao leaf disk assay	Disease suppression through induced systemic resistance	Melnick et al. (2008)
Quercus fusiformis	*Bacillus* spp. *Pseudomonas denitrificans* *Pseudomonas putida*	Reduced incidence, by 50%, of oak wilt disease caused by *B. fagacearum*	Inhibited *B. fagacearum* through chitinase activity	Brooks et al. (1994)
Olea europaea	*Pseudomonas flourescens* PICF7	Reduction in *Verticillium* wilt disease incidence by 82% and disease severity by 96%	Enhancement of plant growth and induced systemic resistance	Mercado-Blanco et al. (2004)
Vitis vinifera	*Pseudomonas* sp. I2R21 *Pseudomonas* sp. W1R33	Reduced by up to 52% the length of trunk lesions caused by Botryosphaeriaceous sp.	Growth inhibition by secondary metabolites	Wicaksono et al. (2017)
Pinus radiata	*Bacillus amyloliquefaciens* *B. Stearothermophilus*	Inhibited the mycelial growth *Macrophomina phaseolina*, causal agent of charcoal disease in *Pinus radiata.*	Growth inhibition by secondary metabolites	Valiente et al. (2008)

Table II-2. Studies of endophytic bacteria in woody plants and their potential as growth promoters or biological controls for tree diseases (continued)

Plant	Endophytic bacteria	Effect on the plants	Modes of action	References
Coffea arabica	*Bacillus lentimorbus* *Bacillus cereus*	Inhibited growth of *Hemileia vastatrix*, causal agent of coffee leaf rust, by 63% in leaf assay.	Growth inhibition by secondary metabolites and chitinase	Shiomi et al. (2006)
Populus sp.	*Bacillus pumilus* JK-SX001	Reduced poplar canker disease, caused by *Cytospora chrysosperma, Phomopsis macrospora* and *Fusicoccum aesculin*, by more than 70% in greenhouse trials	Production of lytic enzymes, cellulases and protease.	Ren et al. (2013)

II.3. Conclusions.

Ceratocystis species have emerged as very aggressive pathogens following the rapid expansion of commercial plantation estates based on *Acacia* species in the past two to three decades. This has led to significant losses of timber production in tropical, subtropical and temperate regions.

Strategies to manage ceratocystis wilt and canker disease have included timely singling and pruning, avoiding the unnecessary creation of wounds, sanitation and chemical treatments. However, these approaches have been both costly and not completely effective. Ground and/or aerial surveys and monitoring of disease incidence and severity can play a crucial role in preventing epidemics, as these provide essential information for prompting disease control measures. Although not yet in place, a cost-efficient and suitable control strategy that can be applied to extensive growing areas is the deployment of host species which are genetically tolerant or resistant to vascular wilt disease. Endophytic microorganisms also show potential as a promising tool to reduce the impact of fungal diseases in agriculture and possibly forestry.

Both approaches, though, require further research input. Current screening protocols to identify resistant trees are slow and laborious; thus, development of rapid screening protocols is needed. Biological control through the use of endophytic bacteria is also an option, but only through their capacity to induce systemic resistance (ISR); this in itself remains a challenge!

III. A Rapid Protocol for Tolerance Screening of *Acacia* species Against *Ceratocystis manginecans* M. van Wyk, Al-Adawi & M.J. Wingf.

Abstract

Ceratocystis wilt and canker disease has had a devastating impact on *Acacia mangium* plantations in SE Asia. Research efforts are directed at developing resistant germplasm, but current screening methods are time-consuming, causing delays in the development of resistant material. In this study, two rapid screening protocols were compared to the current standard practice of inoculating potted plants in the greenhouse.

Inoculation procedures were tested on three species of *Acacia*; *Acacia mangium*, *A. crassicarpa* and an *Acacia* hybrid. Two cultures of *Ceratocystis manginecans* obtained from different geographic regions were used in the experiments. Mycelial plugs were compared with spore suspensions as inoculum to infect artificial wounds on *Acacia* seedlings. The rapid screening protocols included inoculation of stem segments (using mycelial plugs) and detached phyllodes (using a spore suspension). Lesion length on stems and necrosis length on phyllodes were assessed and used as a measure of the susceptibility of the tested plant. The relative levels of susceptibility in each protocol were compared as a basis to assess the utility of the rapid screening protocols. There was a significant difference between the two *Ceratocystis* isolates, however no differences were observed after rejuvenating one isolate by inoculating into and re-isolating from a living tree. Both mycelial plugs and spore suspensions produced a similar level of disease incidence, so either can be used for inoculation assay. The stem segments were prone to contamination by other fungi and to desiccation, while results from the potted plant and phyllode protocols showed similar trends of susceptibility among the *Acacia* clones and

species. The ease, rapidity and reproducibility of the phyllode inoculation protocol makes this a potential replacement for inoculation of potted plants as a preliminary screening protocol to identify *A. mangium* germplasm that is less susceptible to *Ceratocystis* wilt and canker disease.

III.1. Introduction

In Southeast Asia *Eucalyptus* and *Acacia* species have been planted over ~25.6 million hectares of forest plantations, mainly in China, India, Indonesia, Malaysia, Thailand and Vietnam (Harwood & Nambiar 2014b). These fast-growing exotic hardwoods have been established to meet the demand for raw materials for pulp and paper production which, on a global scale, rose substantially from 280 to 500 million tons per annum between 1980 and 2008 (Asia-Pacific Forestry 2010). The more recent reduction in demand for writing paper in mature markets is offset by the increase in writing paper demand in other markets, especially in Asia (Wright 2014).

Despite promising yields in early rotations (Harwood & Nambiar 2014a) disease problems are emerging in these exotic hardwood plantations which seriously impact wood productivity especially in *Acacia mangium* Willd. (Nambiar & Harwood 2014). A root rot caused by the native pathogen *Ganoderma philippii* (Bres. & Henn. ex Sacc.) Bres. can significantly reduce *A. mangium* productivity especially in later rotations (Francis et al. 2014; Mohammed et al. 2014). A recently observed vascular wilt and canker disease caused by *Ceratocystis manginecans* in countries such as Indonesia, Vietnam and Malaysia is of particular concern and is causing large scale mortality of *A. mangium* (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). With productivity nearly halved due to *Ganoderma* and *Ceratocystis*, the industry is shifting, on mineral soils, from planting *A. mangium* to eucalypts (Nambiar & Harwood 2014).

The pathogen that causes canker and wilt disease of *A. mangium* in Indonesia was described as a new species, *C. acaciivora* Tarigan & M. van Wyk (Tarigan et al. 2011a), but was subsequently reduced to synonymy with *C. manginecans* M. van Wyk, Al-Adawi & M.J. Wingf. (Fourie et al. 2015) an important pathogen of especially mango trees (Van Wyk et al. 2007; Al Adawi et al. 2014). Other authors consider that several recently described new species, including *C. acaciivora* and *C. manginecans* are populations within a large species complex for which the most appropriate name is *C. fimbriata* Ellis & Halst. (Oliveira et al. 2016). Authors of a recent population study of *C. manginecans* isolates from SE Asia and Oman concluded that SE Asia may be the centre of origin for *C. manginecans* as the population is more diverse than that in Oman (Fourie et al. 2016) and may have adapted to *A. mangium* from a native host. South American isolates of *C. fimbriata* were not included in that study. As *C. fimbriata* has a very broad host range and not all isolates infect all hosts, in this thesis we use the name *C. manginecans* though acknowledge the likelihood of further taxonomic changes in the near future.

Tree pathogens in the genus ceratocystis are commonly spread via spore transmission from infected trees to healthy trees and use wounds created by animals, humans or insects as their entry points (Kile 1993; Harrington 2013). Spores are often dispersed by wood and bark inhabiting beetles which are attracted to the exudations from infected trees (Heath et al. 2009a). Spores in frass may also be transported on air currents and infect wounds (Harrington 2007). A key strategy to prevent *Ceratocystis* transmission among trees is to avoid wounding (Kile 1993) or minimize the size of pruning wounds, prune carefully and conduct pruning operations during the seasons when insect vectors are less active (Heath et al. 2009a; Tarigan et al. 2011b). When wounding cannot be avoided, a wound dressing is highly recommended for preventing *Ceratocystis* infection of trees (Harrington 2013). These type of management strategies are costly and ineffective for large scale acacia plantations especially as *Acacia* species requires

singling and is prone to monkey or squirrel damage (Tarigan et al. 2011b). The use of genetic resistance by selection and breeding of disease tolerant or resistant materials is considered as the most cost effective and durable method for controlling *Ceratocystis* wilt disease in tropical *Acacia* plantations (Roux & Wingfield 2009). It is useful to distinguish between resistance - attributes that prevent trees being infected by a disease; and tolerance - the ability of the tree, once infected, to continue to grow and survive (Brawner et al. 2015). Selecting and breeding to reduce damage from *C. manginecans* potentially will involve genetic improvement in several independent resistance and tolerance traits.

Levels of tolerance to *C. manginecans* in *A. mangium* are low and resistance is rarely observed (Brawner et al. 2015). Therefore, large numbers of plants must be screened to obtain a few tolerant plants for breeding programs (Brawner et al. 2015). Other species such as *Acacia crassicarpa* Cunn. ex Benth. and *Acacia auriculiformis* Benth. show greater tolerance to *C. manginecans* (Harwood, personal communication). The gradient of tolerance to *C. manginecans* found in other species of *Acacia*, especially *A. auriculiformis* must be fully exploited in transferring tolerance to *A. mangium* through hybridisation (Harwood personal communication).

The current procedure for the preliminary screening of *Acacia* trees against *C. manginecans* is by inoculation of seedlings or clonal cuttings in the greenhouse. However, this process tends to be slow due to the large number of plants which must be screened, and the length of time plants must be maintained before disease levels can be assessed. Therefore, a more rapid screening process has the potential to expedite breeding programs (Wingfield et al. 2001a).

Few reports are available about rapid procedures to evaluate tolerant or resistant plants against *Ceratocystis* species. For instance, five potential procedures to evaluate the resistance of *Eucalyptus* species against *Ceratocystis fimbriata sensu lato (s.l.)* were carried out by Van

Wyk et al. (2010); including *in vitro* growth comparisons on artificial media, inoculation of *C. fimbriata s.l.* on apples, eucalypt seedlings in a greenhouse, eucalypt bolts freshly cut from stems of young trees; and field inoculations on young trees were compared. Inoculating eucalypt bolts gave similar results to field inoculations, required less space and meant that the pathogen was exposed to the environment. Recent research by Magalhães et al. (2016) reported that data obtained from inoculating cacao leaf discs with *Ceratocystis cacaofunesta* Engelbrecht & T.C. Harr. reflected the field resistance of cacao trees against wilt disease caused by this fungal pathogen. This method of screening cacao leaf material for *Ceratocystis* resistance may be applicable to *Acacia* species.

The aim of this study was to improve procedures used to screen *Acacia* species for resistance to *C. manginecans* by comparing the current screening protocol (inoculation of potted plants) with two potential rapid screening procedures; namely inoculation of *Acacia* phyllodes and stem segments. We also evaluated whether the expression of resistance or pathogen virulence is influenced by the type of inoculum (mycelium or spore suspension).

III.2. Materials and Methods

III.2.1. Plant material

Acacia mangium is grown on mineral soils and *Acacia crassicarpa* on peat soils in Sumatra. Clones being planted in Riau Province, Sumatra by Riau Andalan Pulp & Paper (RAPP) at the time of the study were selected; five of *A. mangium* (RPT00021, RPT00316, RPT00868, RPT00870 and RPT00887) and two of *A. crassicarpa* (RPT30003 and RPT 30020). *Acacia mangium* is reported as a species very susceptible to *C. manginecans* (Brawner et al. 2015), and the species *A. crassicarpa* as being less susceptible to this pathogen (Tarigan et al. 2013). Acacia hybrids between *A. auriculiformis* x *A. mangium* are not yet commercially

deployed in Indonesia by the large pulp and paper companies but are grown commercially in Vietnam and have been shown to have a range of susceptibility (Thu et al. 2012; Trang et al. 2018). In this study two *Acacia* hybrid clones (RPX80498 and RPX80537) being tested commercially by RAPP were selected.

III.2.2. Fungal isolates

The two cultures of *C. manginecans* used in this study were isolated from *A. mangium* showing symptoms of ceratocystis wilt. Isolate CTA-128 and CTA-138 originated from two different RAPP estates, Teso and Logas respectively, both located in Pekanbaru, Riau Province, Sumatra, Indonesia. Both cultures were isolated in 2012 and identified as *C. acaciivora*, CTA-128 was also accessioned as CMW22562 and CTA-138 as CMW22595 (Tarigan et al. 2011a). To compare the pathogenicity of an isolate when freshly isolated and stored for 4 years, CTA-138 was inoculated into and re-isolated from an *A. mangium* seedling three weeks prior to its use, and then grown on malt extract agar (MEA) for 7 days at 22 °C. The freshly isolated culture derived from CTA-138 was differentiated as CTA-138*.

III.2.3. Potted plant inoculation protocol

Acacia cuttings were rooted in the nursery for 6 weeks and then planted in polybags 20 x 30 cm in size. Mineral top soil and 10 grams of Osmocote®, a slow release fertilizer, were added to each bag. The plants in polybags were set out in an open area dedicated to pot trials and adjacent to greenhouses at RAPP headquarters in Kerinci, Riau Province. All seedlings were maintained for 3 months before inoculating with *C. manginecans*.

Inoculation of potted plants was carried out following a previously published protocol (Van Wyk et al. 2010). The stems were wounded approximately 200 mm above soil level using a sterile scalpel. Mycelium for inoculum was taken from the edge of the culture plate to ensure it was actively growing. The *C. manginecans* plug was then placed into the wound by facing

the pathogen mycelium against the cambium and covering with parafilm. Conidial suspensions for inoculum were prepared by harvesting 7 day old *C. manginecans* cultures of CTA-138, CTA-138* and CTA-128 following Baker's procedure (Baker et al. 2003). The culture was flooded with 10 ml sterile distilled water and then scraped with a sterile spatula in order to suspend the spores. The suspension was then sieved using a sterile millipore cloth (Miracloth Sigma Aldrich) and the concentration of conidia estimated using a haemocytometer. Conidial suspensions were diluted to a concentration of $2x10^5$ conidia per millilitre and a 10 µl aliquot was pipetted into the wound in the stems. Control treatments of sterile agar plugs or sterile distilled water were applied as for fungal inoculum treatments. In order to avoid contamination and desiccation, the treated wounds were then sealed with Parafilm.

Inoculated seedlings were maintained for 3-5 weeks and monitored for disease symptoms. Lesions were indicated by sapwood discoloration which shows as dark reddish brown to purple discoloration in both external (bark) and internal (sapwood) tissues. External lesions were recorded weekly starting from three weeks after inoculation (WAI) to five WAI, at which time stems were debarked and internal lesions were measured. An internal or external discoloration index (DI) was then calculated as the percentage of lesion length per total stem length.

III.2.4. Stem segment inoculation protocol

Stem segments (approximately 35 cm length by 3 cm diameter) were harvested from the main stem of 6-month-old potted plants of the *Acacia* species and clones to be screened. Tests to avoid desiccation and contamination in the stem segment procedure showed that covering both ends of the stems with wax reduced drying and contamination compared with covering the ends of the stems with moist cotton, paper tape or sitting the segments in moist sand. The waxing of stem segment ends slowed desiccation for at least 65% of the stems but only for a maximum of 3 weeks after inoculation. When the inoculated stem segments were

placed in sterile clear plastic bags to further slow desiccation, contaminant fungi quickly obscured the surface of the lesions and it was difficult to assess external lesion development beyond two WAI.

Prior to inoculation, the stem segments with waxed ends were wounded using a sterilized cork borer (0 5-mm diameter). Then a plug of *C. manginecans* mycelium was taken from the edge of the culture and inserted with mycelium facing directly into the wound. The inoculated wound was then covered with paper wrapping tape (Kingstone masking tape, 24 mm wide). Sterile agar plugs were applied in the same manner to control samples. Inoculated and control stem segments were incubated at room temperature (22–25°C) on the laboratory bench for 3 weeks. External lesions were recorded every 7 days after inoculation for 3 weeks. Internal lesions were measured in the third week by removing the bark of the stem segment. A discolouration index (DI) was calculated as the percentage of longitudinal discolouration per total stem segment length.

III.2.5. Phyllode inoculation protocol

Preliminary tests were carried out with phyllodes which were collected from young trees planted in the soil in the resistance screening area adjacent to the greenhouses at RAPP, Kerinci, Sumatra. Phyllodes of different ages were collected and inoculated but there was little difference in the lesion development between phyllodes of different ages (data not shown). The phyllode bases were quickly wrapped in moist cotton and the maximum storage time before inoculation was 24 hours at 4 ºC in a plastic bag. If this storage time was exceeded, desiccation or phyllode tip browning was observed.

Inoculum was prepared as described for the inoculation of potted plants with conidia. A 10 µl aliquot was pipetted into the middle of the surface of the phyllode after wounding with a sterile needle. Phyllodes which were used as controls received 10 µl of sterile distilled water

in place of the conidial suspension. The phyllodes were put into clear, sterile, plastic bags with sterile, moistened cotton to avoid desiccation and maintained at 25^0C until the disease symptoms appeared.

The length of the lesion and discolouration in phyllodes was observed and measured daily for up to two weeks after inoculation. A discolouration index (DI) was calculated as the percentage of discolouration length per total phyllode length.

III.2.6. Comparison of screening protocols

Three screening inoculation procedures were compared in two experiments based on (1) five different *A. mangium* clones, RPT00021, RPT00316, RPT00868, RPT00870 and RPT00887 and (2) three *Acacia* species, *A. mangium, A. crassicarpa* and *A.* hybrid. The three inoculation procedures were; inoculation of potted plants using mycelial plugs and conidial suspension, inoculation of detached stem segments and inoculation of detached phyllodes.

In the **first experiment**, five clones of *A. mangium* namely RPT00021, RPT00316, RPT00868, RPT00870 and RPT00887 were used for comparing potted plants, stem segment and phyllode inoculation protocol. All five *A. mangium* clones were inoculated with *C. manginecans*.

The potted plant trial was set up as a randomized complete block design (RCBD) with 5 *A. mangium* clones and 5 replicate blocks. There were 8 treatments with 20 plants in each experimental unit, and 800 plants for the total experiment. Different fungal inoculum types were also compared in this first experiment with potted plants and the 6 fungal inoculation treatments were mycelial plugs of each of the three *C. manginecans* isolates (CTA-138*, CTA-138 and CTA-128) and conidial suspensions of each of the same three isolates. The 2 control treatments were inoculated with either sterile water agar plugs or sterile distilled water. For the stem segment protocol with the 5 clones the inoculation treatments consisted of mycelium of

3 isolates CTA-128, CTA-138, CTA 138* and sterile water agar plugs as controls. Each treatment was applied to 20 stem segments. For the phyllode protocol the 5 clones were inoculated with conidial suspensions of CTA-138*, CTA-138 and CTA-128. Sterile distilled water was used as a control treatment. There were 20 phyllodes per treatment.

In a **second experiment** the responses to *C. manginecans* of two clones each of 3 species were compared between the potted plant, stem segment and phyllode inoculation protocols; *A. mangium* (RPT00021 and RPT00316), *Acacia crassicarpa* (RPT30003 and RPT 30020) and *Acacia* hybrid (*A. auriculiformis* x *A. mangium*) (RPX80498 and RPX80537).

The potted plant trial was set up in a randomized complete block design (RCBD) with the 6 clones and 5 replicate blocks for each treatment. Two different fungal inoculum types of a single isolate of *C. manginecans* were compared in this second experiment with potted plants. The isolate was selected based on its aggressiveness or high virulence in the previous inoculation assay. The four treatments were 2 types of inoculum (mycelium and conidia) from CTA-138*, 2 controls (sterile water agar and sterile distilled water). There were 20 plants per experimental unit i.e. a total of 480 plants per block. For the stem segment protocol with the 6 clones the inoculation treatment consisted of the mycelium of CTA 138* with sterile agar plugs as control and for phyllode protocol the inoculation treatment was a conidial solution of CTA 138*with sterile distilled water as control. Each treatment was applied to 20 phyllodes and 20 stem segments from each of the 6 clones.

III.2.7. Statistical analysis

Differences in discolouration indices between different *Acacia* clones and species in response to mycelial and conidial inoculum, stored and newly re-isolated cultures, and the variation in time until the first appearance of symptoms for each of the screening protocols were analysed for each of three inoculation procedures by means of repeated measures analysis

of variance (ANOVA) using the general linear model (GLM) procedure of SAS (SAS system v 9.4; SAS Institute Inc., Cary, NC, USA) and Fisher's LSD test.

The results obtained from the three different inoculation protocols (potted plants, phyllodes and stems segments) were compared using correlation analysis.

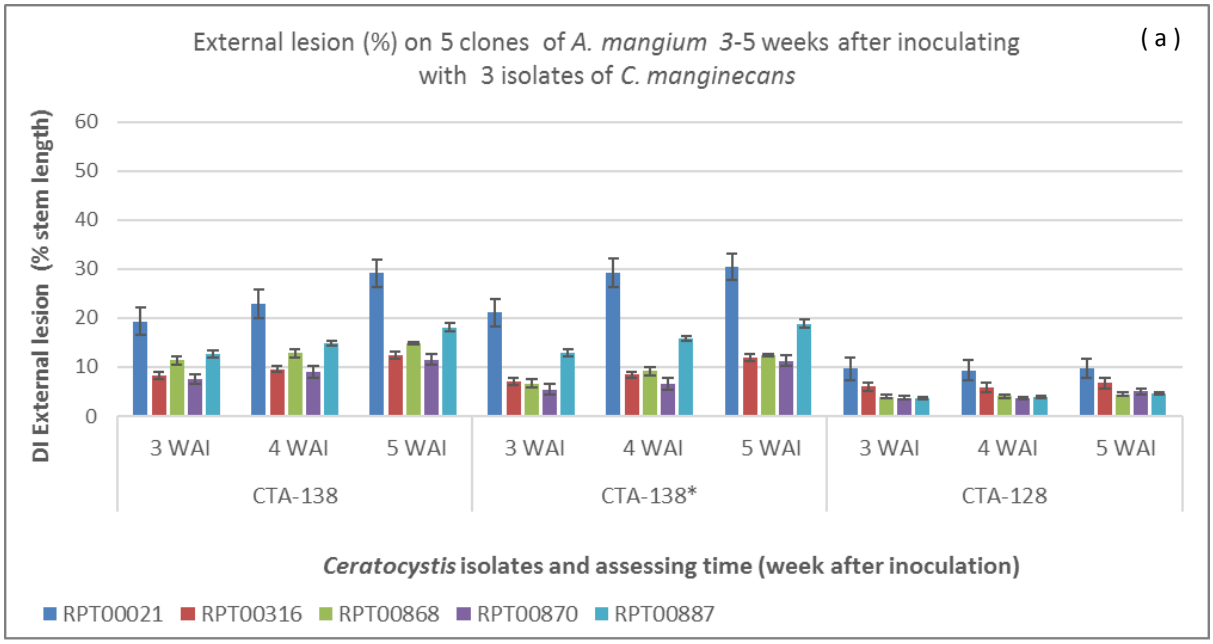
III.3. Results

III.3.1. Lesion development

III.3.1.1. Potted plants

The discolouration or sap streaks on inoculated stems of potted *Acacia* seedlings first appeared 7-14 days after inoculation with either mycelial plugs or conidial suspensions of *C. manginecans*. No lesions emerged on stems which were inoculated with sterile distilled water or sterile water agar plugs. In experiment 1 external DIs varied among the five *A. mangium* clones irrespective of the isolate inoculated although inoculation with isolate CTA-128 resulted in the smallest external DIs (Figure III-1a). With isolate CTA-138* the mean external DI for clone RPT00021 by 5 weeks after inoculation was approximately 30% of total stem length (Figure III-1a).

At 5 WAI, internal Dis Figure III-1b were 1.5 to 2 times larger than external DIs at 5 WAI. Seedlings began to wilt at week 4 and phyllodes also started to yellow. The majority of RPT00021 seedlings were wilting by 4 WAI and were dry and dead by 5 WAI. However, internal lesions on stems of seedlings that had died prior to 5 WAI were still clear and measurable.



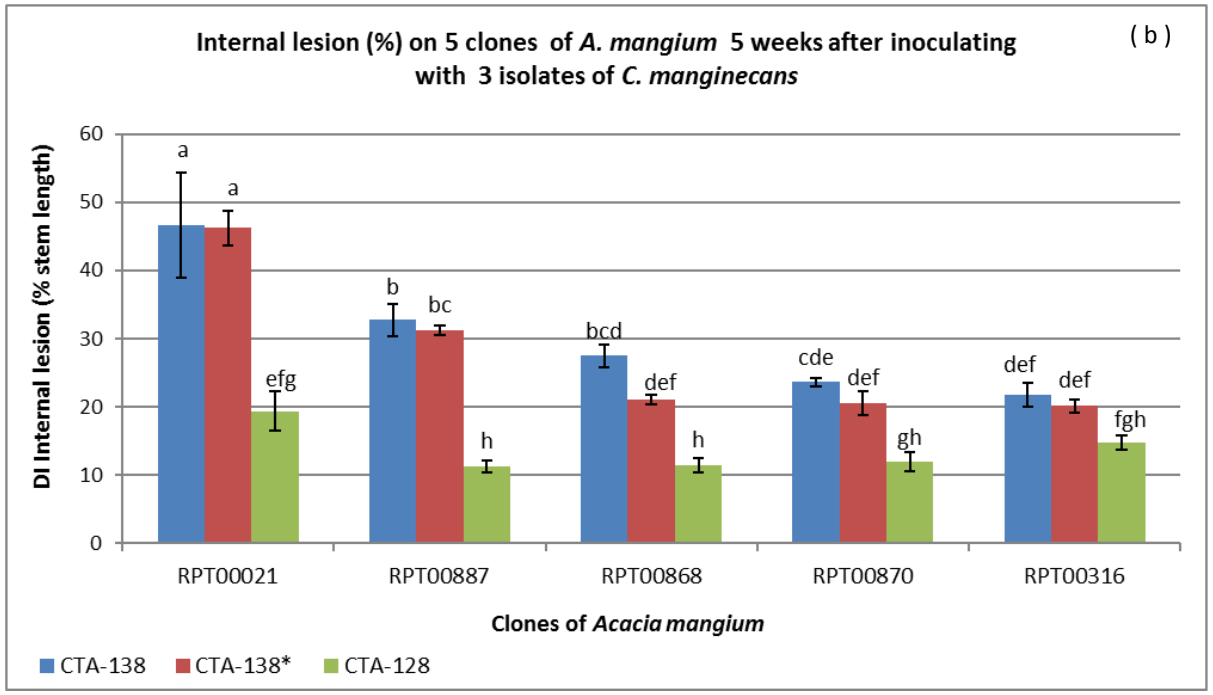


Figure III-1. Mean discolouration length per total stem length (DI values) based on (a) external lesions on five clones of *A. mangium* at 3, 4 and 5 weeks after inoculating potted plants with mycelium plugs from three isolates of *C. manginecans* (CTA-138, CTA-138* and CTA-128), (b) mean DI values based on internal lesions at 5 weeks after inoculation with three cultures (CTA-138, CTA-138* and CTS-128). Different letters (a-h) indicate statistically significant differences between clones (Fisher's LSD test, $p<0.05$, bars represent standard errors of mean DI value).

In experiment 2, the growth of external lesions was greater on *A. mangium* clones RPT00021 compared with all other clones and species (Figure III-2a,b). The external DIs of RPT00021 increased from 12.4% at 3 WAI to up to 35% by 5 WAI, significantly higher than the other five tested clones (Figure III-2a). Most of RPT00021 seedlings (> 50%) were yellowing and wilting at 4 WAI with desiccation and death occurring by 5 WAI.

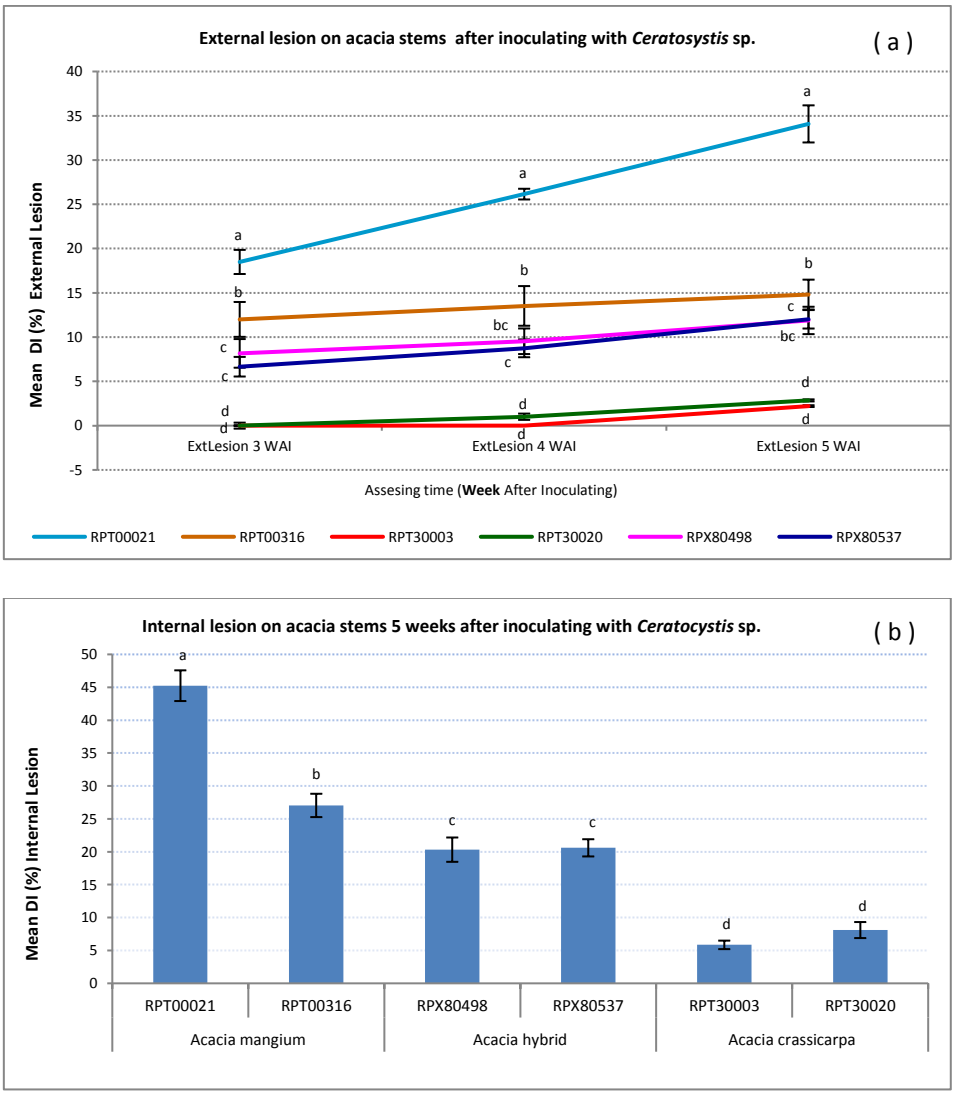


Figure III-2. Mean DI values based on external lesion at 3,4 and 5 weeks (a) and internal lesion after 5 weeks (b) on *A. mangium* (RPT0021 and RPT00316), *A. crassicarpa* (RPT3003 and RPT30020) and *A.* hybrid (RPX80498 and RPX80537) after inoculating potted plants with *C. manginecans* isolate CTA-138*. Different letters (a-d) indicate statistically significant differences between clones and species (Fisher's LSD test, $p < 0.05$, bars represent standard errors of mean DI value).

Both experiment one and two showed differences between external and internal lesion behaviours in *Acacia* stems, where the external lesion did not always appear continuous although the internal lesion was always continuous (Figure III-3a, b).

Figure III-3. The discoloration on acacia stems caused by *Ceratocystis manginecans*. External discoloration was not continuous (a), internal lesion growth along the acacia stem (b).

III.3.1.2. Stem segments

The external lesions on five tested clones of *A. mangium* first appeared 7 to 14 days after inoculation. Even though both tips of stems were covered with wax, the stems were still prone to desiccation. Other contaminant fungi also emerged on the stem surfaces. These two problems contributed to slower lesion development on the stem, obscured the disease symptom and thus limited the duration of external lesion measurement to a maximum of two weeks.

The analysis of variance among five tested *A. mangium* clones on this stem inoculation method resulted in a significant (LSD test, $p<0.05$) difference in DI, with RPT00887 the most

susceptible and RPT00868 the least. Both analyses on external and internal lesion produced almost the same results (Figure III-4a, b). Following this stem protocol, the internal lesion was also longer than the external lesion, as with the potted plants. The lesions were observed more clearly internally than externally due to external stem surface contamination.

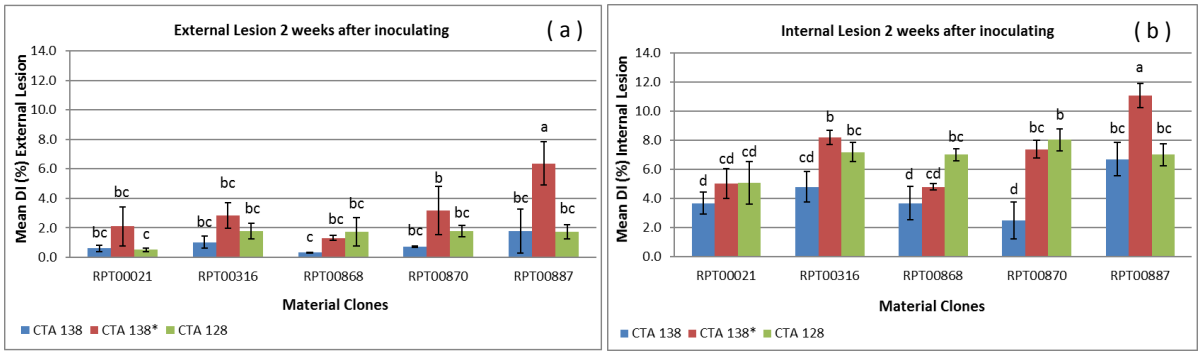


Figure III-4. Mean DI values on five *Acacia mangium* clones 14 days after inoculating branch segments with three isolates of *C. manginecans* (CTA-138, CTA-138* and CTA 128), based on (a) external lesions, (b) internal lesions. Different letters (a-d) indicate statistically significant differences between clones (Fisher's LSD test, $p < 0.05$, bars represent standard errors of mean DI value).

Experiment two revealed that *Acacia crassicarpa* was less susceptible to *Ceratocystis* infection compared with *A. mangium* and *Acacia* hybrid. As in experiment I, external lesions first appeared 7 days after inoculation. Fungal contamination on the stem segment surfaces was avoided by not placing the inoculated stems in plastic bags, however, the stems still tended to dry after 2 WAI.

The two *A. crassicarpa* clones (RPT30003 and RPT30020) in this experiment had significantly smaller DIs (LSD test, $p<0.05$) compared to all the other clones (Table III-1). Meanwhile, *A. mangium* RPT0021 was the most susceptible and RPT00316 the second most susceptible. The *A.* hybrid clones were intermediate between *A. crassicarpa* and *A. mangium*, with RPX80537 not significantly different from the two *A. crassicarpa* clones and RPX80498 not significantly different from *A. mangium* clone RPT00316.

Table III-1. Discolouration indices (DIs) of external and internal lesions on branch segments for six *Acacia* clones 14 days after inoculating with *C. manginecans* isolate CTA-138*. Different letters (a-d) within a column indicate statistically significant differences between clones (LSD test, p <0.05).

Plant		External Lesion (%)	Internal Lesion (%)
Species	Clone	14 DAI	14 DAI
A. mangium	RPT00021	7.4 ± 0.5^{a}	7.8 ± 0.5^{a}
A. mangium	RPT00316	5.8 ± 0.5^{b}	6.1 ± 0.5^{b}
A. crassicarpa	RPT30003	2.5 ± 0.5^{d}	3.1 ± 0.5^{d}
A. crassicarpa	RPT30020	2.6 ± 0.5^{d}	3.1 ± 0.5^{d}
A. hybrid	RPX80498	4.8 ± 0.5^{bc}	5.3 ± 0.5^{bc}
A. hybrid	RPX80537	3.7 ± 0.5^{cd}	4.5 ± 0.5^{cd}

*) = Day after inoculation

Number in column followed by the same letter are not statistically different at p<0.05

III.3.1.3. Phyllodes

Necrotic symptoms on phyllodes appeared 5 days after inoculation. The necrosis increased gradually and, in susceptible clones, reached the phyllode edge within 2 weeks of the first appearance of symptoms (Figure III-5 a, b & c).

Figure III-5. Necrotic development on *A. mangium*, (a) 5, (b) 10 and (c) 14 days after inoculating with *Ceratocystis manginecans* (CTA-138*).

The analysis of variance among five tested clones of *A. mangium* showed that there was a significant difference between clones with the lowest DI in RPT00316 and the highest in RPT00870 ($p < 0.05$, Figure III-6). The ranking of phyllode responses remained stable until 19 days after phyllodes were inoculated. The DI values using the other two isolates (CTA 138 and CTA 128) also demonstrated the same trend with lowest DIs in clones RPT00316 and RPT00870.

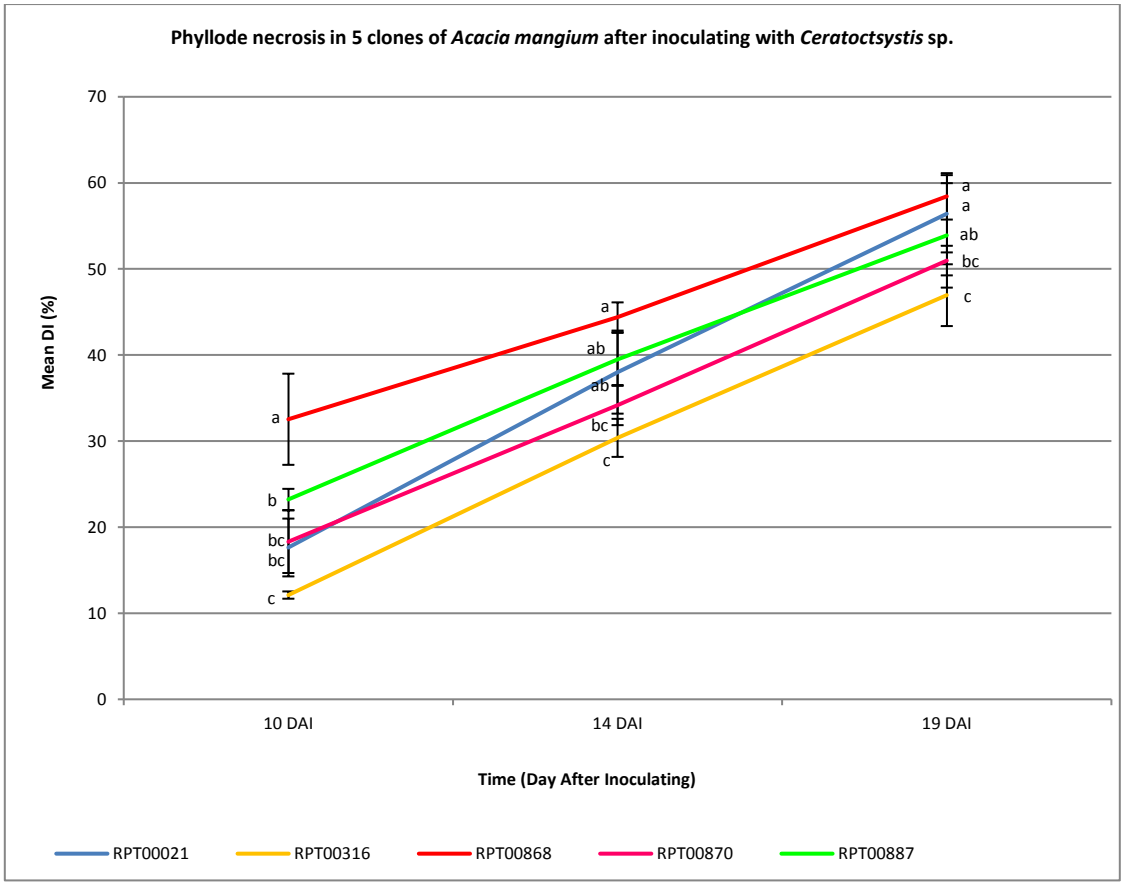


Figure III-6. Mean discolouration indices (DI value), based on proportion of necrotic length per phyllode length on five *Acacia mangium* clones 10, 14, and 19 days after inoculating with *C. manginecans*, isolate (CTA-138*). Different letters indicate statistically significant differences among clones (Fisher's LSD test, $p < 0.05$, bars represent standard errors of mean DI value).

Acacia crassicarpa exhibited the lowest degree of necrosis compared with *A. mangium* and *Acacia* hybrid in the second phyllode experiment. While clone RPT3003 had a significantly lower (LSD test, $p<0.05$) DI than RPT30020, both were significantly lower than clones of *A. mangium* and *A.* hybrid (Figure III-7). *A. mangium* clone RPT0021 had the highest mean DI and RPT00316 the second highest. Meanwhile, *A.* hybrid clones (RPX80498 and RPX80537) were intermediate between the two parent species, and significantly ($p<0.05$) different at 19 DAI.

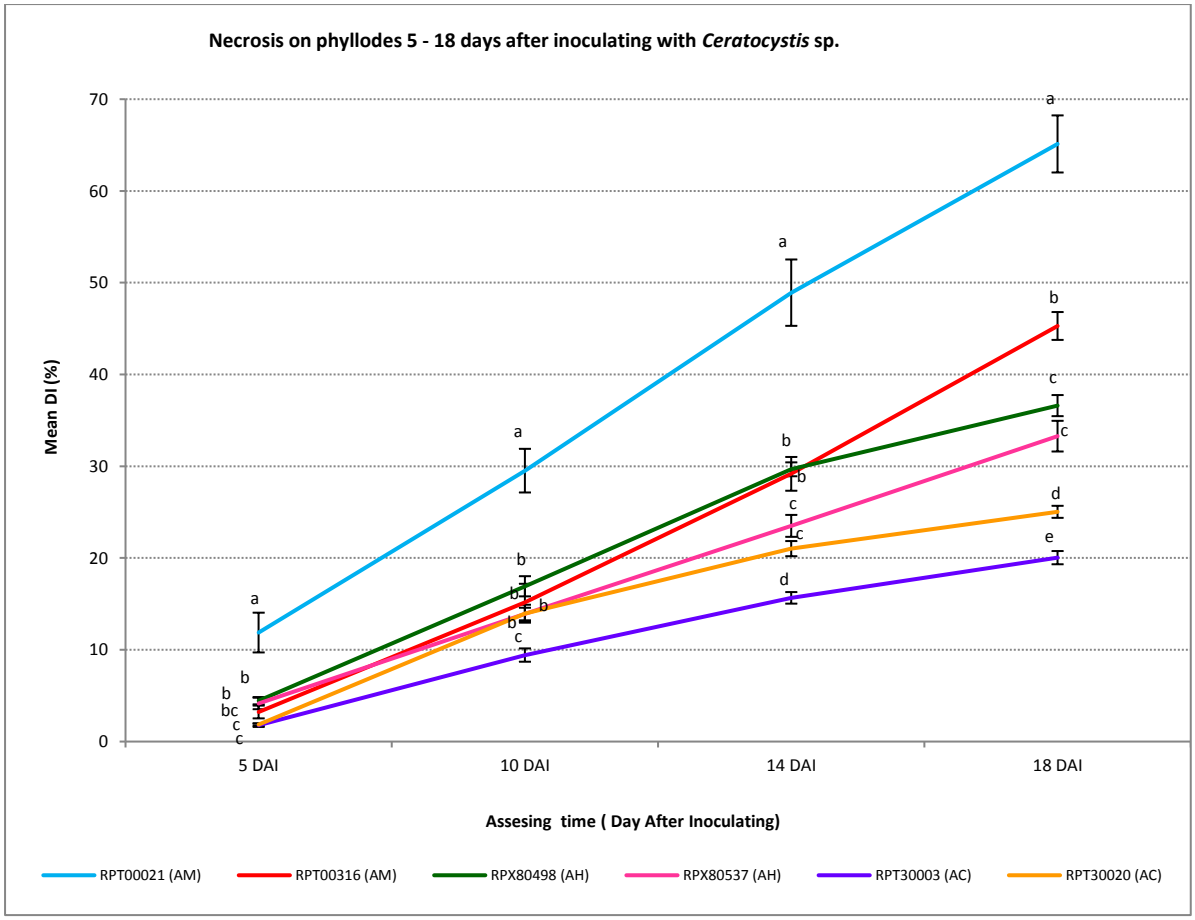


Figure III-7. Mean discolouration indices (DI value), based on proportion of necrotic length per phyllode length on *Acacia mangium* (RPT0021 and RPT00316), *Acacia crassicarpa* (RPT3003 and RPT30020) and *Acacia hybrid* (RPX80498 and RPX80537) at 5, 10, 14, and 18 days after inoculating with *C. manginecans*, isolate CTA-138*. Different letters (a-e) indicate statistically significant differences among clones at each assessment date (Fisher's LSD test, $p < 0.05$, bars represent standard errors of mean DI value).

III.3.2. Spores versus mycelium

In both experiments and irrespective of *Acacia* species or clone, inoculation with spores had lower discolouration indices (DIs) compared with mycelium plugs in the early assessments, but not in later assessments of either internal or external lesions (Table III-2).

Table III-2. Mean discolouration indices (DIs) for stems of potted plants of five clones of *A. mangium* (A) and six clones of *A. mangium*, *A. crassicarpa* and *Acacia* hybrid (B) at 3, 4 and 5 weeks after inoculating with three isolates of *C. manginecans* (CTA-138, CTA-138* and CTA-128). DIs are expressed as mean values ± standard error (SE). Different letters (a-b) on the same assessment date indicate statistically significant differences between the two inoculum types, mycelium and spore suspension (Fisher's LSD test, $p<0.05$). WAI= Weeks after inoculation.

A. Inoculating five clones of *Acacia mangium*

Inoculum	Mean DI (%) external			Mean DI (%) internal
form	3 WAI	4 WAI	5 WAI	5 WAI
Mycelium	10.8 ± 0.7[a]	12.5 ± 1.0[a]	15.4 ± 1.3[a]	26.2 ± 1.7[a]
Spores	7.8 ± 0.7[b]	9.5 ± 1.0[b]	11.8 ± 1.3[a]	21.8 ± 1.7[a]

B. Inoculating *Acacia mangium, Acacia crassicarpa* and *Acacia* hybrid

Inoculum	Mean DI (%) external			Mean DI (%) internal
form	3 WAI	4 WAI	5 WAI	5 WAI
Mycelium	7.7 ± 0.7[a]	9.9 ± 0.8[a]	13.0 ± 0.8[a]	21.2 ± 0.9[a]
Spores	5.6 ± 0.7[b]	9.1 ± 0.8[a]	12.3 ± 0.8[a]	19.1 ± 0.9[a]

III.3.3. Isolates of different origin and period stored in culture

The DIs of potted plants inoculated with two different isolates of *C. manginecans* from different geographic locations were significantly different. CTA-128 gave rise to significantly lower DIs at all assessment dates (LSD test, $p<0.05$) than CTA-138 or the freshly re-isolated CTA-138* (Table III-3, Figure III-8). There was no statistically significant difference between the DIs produced by the two identical *Ceratocystis* isolates CTA-138 (stored in culture) and CTA-138* (freshly re-isolated) at any of the assessment dates (Table III-3).

Table III-3. Comparison of discolouration indices (DIs) for potted *Acacia* clones inoculated with three isolates of *Ceratocystis manginecans*. DIs are expressed as mean values ± standard error (SE). Different letters (a-d) in the same column indicate statistically significant differences between isolates at that assessment (Fisher's LSD test, $p < 0.05$). WAI= Weeks after inoculation.

Ceratocystis	Mean DI (%) external		Mean DI (%) internal	
isolates	3 WAI	4 WAI	5 WAI	5 WAI
CTA - 138	11.8 ± 0.7[a]	13.8 ± 0.8[a]	17.8 ± 1.0[a]	30.4 ± 1.2[a]
CTA - 138*	10.6 ± 0.7[a]	13.9 ± 0.8[a]	17.0 ± 1.0[a]	27.8 ± 1.2[a]
CTA - 128	5.4 ± 0.7[b]	5.4 ± 0.8[b]	6.1 ± 1.0[b]	13.8 ± 1.2[b]

III.3.4. Comparison of potted plant, stem segment and phyllode inoculation protocols

Differences in DI among five *A. mangium* clones inoculated with CTA-128, CTA-138 and CTA-138* were significant at all external lesion assessment dates (data not shown) and at the internal lesion assessment (Table III-4). Internal DIs for isolate CTA-128 were the lowest for all clones (11.3-19.3%). For the other two isolates, RPT00021 had high external DIs (46.3-46.6%) compared to the lower DIs for clone RPT00316 (20.2-21.8%). Clone RPT00877 also had relatively high internal DIs (31.2-32.7%) with CTA-138 and CTA 138*. The DIs of clones RPT00868 and RPT00870 were intermediate between RPT00877 and RPT00316. The phyllode DIs two weeks after inoculation were significantly different with similar trends in respect to the response of the clones to the different isolates as for the inoculation of potted plants (Table III-4) although the DIs had a higher range (33.5-65.9%). This was confirmed by a correlation analysis of the data from potted plant inoculations (Figure III-8). At two weeks after inoculation there were significant differences between the internal DIs for stem segments. However, no clear trend in these differences in respect to the clone or the isolate inoculated

was observed (Table III-4) and no correlation of results with either the potted plant or phyllode inoculation protocols (Figure III-8).

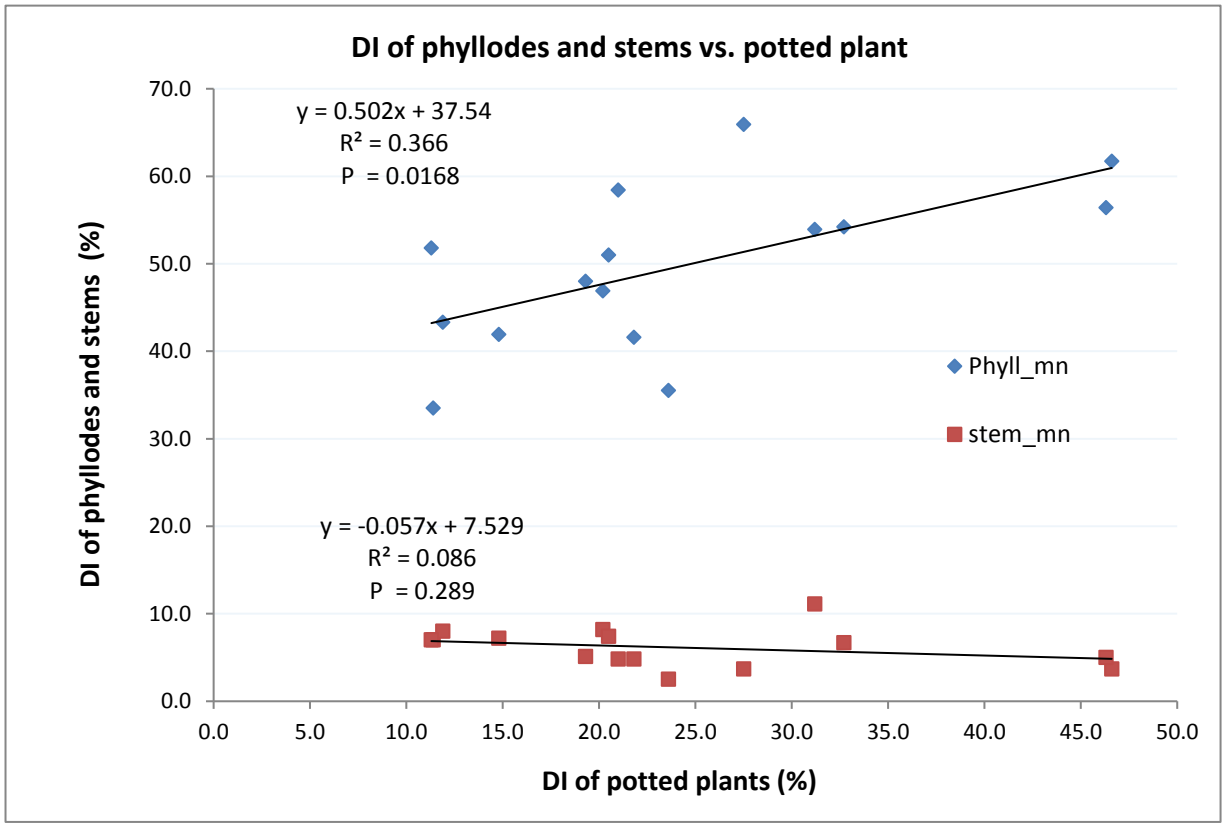
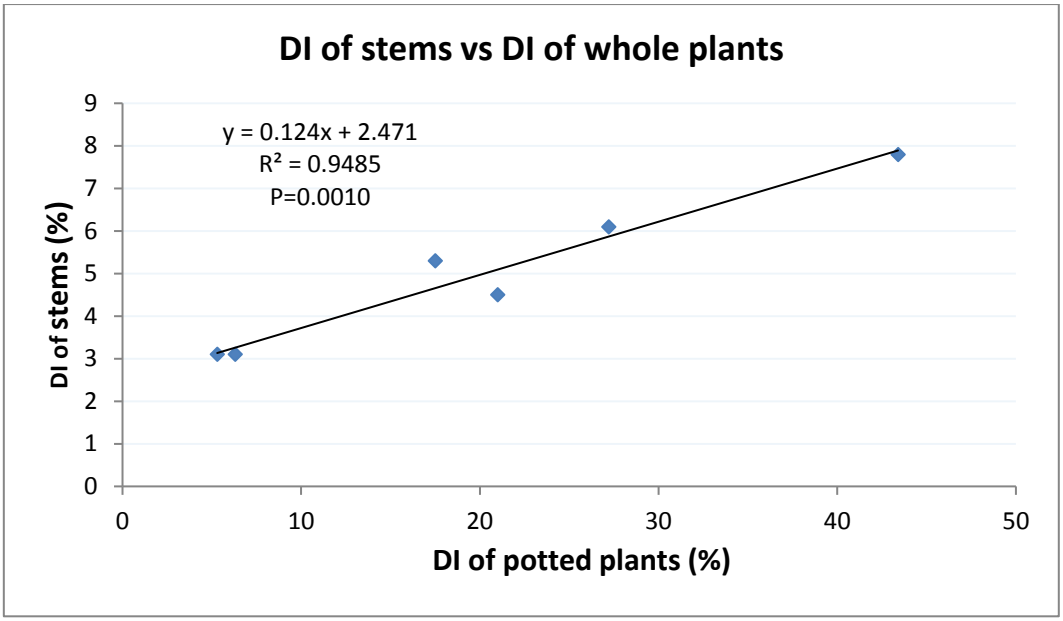


Figure III-8. Regression analysis of phyllode and stem disease index (DI) against potted plant DI for five clones of *A. mangium*. Correlation between phyllode and potted plant inoculations is significant but there is no correlation between inoculation of detached stem segments and potted plants.

Table III-4. Comparison of discolouration indices (DIs) for three inoculation protocols. DIs are expressed as mean values ± standard error (SE). Different letters (a-h) in the same column indicate statistically significant differences between clones and species at that assessment (Fisher's LSD test, $p < 0.05$). WAI= Weeks after inoculation.

POTTED PLANT INOCULATION PROTOCOL			PHYLLODE INOCULATION PROTOCOL			STEM INOCULATION PROTOCOL		
Clone	**Isolate**	**Mean value internal DI (%) 5 WAI**	**Clone**	**Isolate**	**Mean value DI (%) 2 WAI**	**Clone**	**Isolate**	**Mean value internal DI (%) 2 WAI**
RPT00021	CTA-138	46.6 ± 2.7^{a}	RPT00868	CTA-138	65.9 ± 3.5^{a}	RPT00887	CTA-138*	11.1 ± 0.9^{a}
RPT00021	CTA-138*	46.3 ± 2.7^{a}	RPT00021	CTA-138	61.7 ± 3.5^{ab}	RPT00316	CTA-138*	8.2 ± 0.9^{b}
RPT00887	CTA-138	32.7 ± 2.7^{b}	RPT00868	CTA-138*	58.4 ± 3.5^{abc}	RPT00870	CTA-128	8.0 ± 0.9^{b}
RPT00887	CTA-138*	31.2 ± 2.7^{bc}	RPT00021	CTA-138*	56.4 ± 3.5^{abcd}	RPT00870	CTA-138*	7.4 ± 0.9^{bc}
RPT00868	CTA-138	27.5 ± 2.7^{bcd}	RPT00887	CTA-138	54.2 ± 3.5^{bcd}	RPT00316	CTA-128	7.2 ± 0.9^{bc}
RPT00870	CTA-138	23.6 ± 2.7^{cde}	RPT00887	CTA-138*	53.9 ± 3.5^{bcd}	RPT00868	CTA-128	7.0 ± 0.9^{bc}
RPT00316	CTA-138	21.8 ± 2.7^{def}	RPT00887	CTA-128	51.8 ± 3.5^{bcde}	RPT00887	CTA-128	7.0 ± 0.9^{bc}
RPT00868	CTA-138*	21.0 ± 2.7^{def}	RPT00870	CTA-138*	51.0 ± 3.5^{cde}	RPT00887	CTA-138	6.7 ± 0.9^{bc}
RPT00870	CTA-138*	20.5 ± 2.7^{def}	RPT00021	CTA-128	48.0 ± 3.5^{cde}	RPT00021	CTA-128	5.1 ± 0.9^{cd}
RPT00316	CTA-138*	20.2 ± 2.7^{def}	RPT00316	CTA-138*	46.9 ± 3.5^{de}	RPT00021	CTA-138*	5.0 ± 0.9^{cd}
RPT00021	CTA-128	19.3 ± 2.7^{efg}	RPT00870	CTA-128	43.3 ± 3.5^{ef}	RPT00316	CTA-138	4.8 ± 0.9^{cd}
RPT00316	CTA-128	14.8 ± 2.7^{fgh}	RPT00316	CTA-128	41.9 ± 3.5^{ef}	RPT00868	CTA-138*	4.8 ± 0.9^{cd}
RPT00870	CTA-128	11.9 ± 2.7^{gh}	RPT00316	CTA-138	41.6 ± 3.5^{ef}	RPT00021	CTA-138	3.7 ± 0.9^{d}
RPT00868	CTA-128	11.4 ± 2.7^{h}	RPT00870	CTA-138	35.5 ± 3.5^{f}	RPT00868	CTA-138	3.7 ± 0.9^{d}
RPT00887	CTA-128	11.3 ± 2.7^{h}	RPT00868	CTA-128	33.5 ± 3.5^{f}	RPT00870	CTA-138	2.5 ± 0.9^{d}

A high correlation among phyllode, stem and potted plant protocols was obtained when clones of *A. mangium* (RPT00316 and RPT00021), *A. crassicarpa (*RPT30003 and RPT30020) and *A.* hybrid (RPX80537 and RPX80498) were inoculated with CTA-138* (Table III-5, Figure III-9). The two *A. mangium* clones RPT00316 and RPT00021, selected as the least and most susceptible clones from the experiment with five *A. mangium* clones, were again significantly different from each other for all inoculation protocols. Both the *A. mangium* clones however had significantly higher DIs than the *A. crassicarpa* and *Acacia* hybrid clones (LSD test, $p<0.05$). For all inoculation protocols the two *A. crassicarpa* clones (RPT30003 and RPT30020) had significantly smaller Dis ($p < 0.05$) compared to all the other clones (Table III-5). The DIs for the *Acacia* hybrid clones were intermediate between *A. crassicarpa* and *A. mangium*.



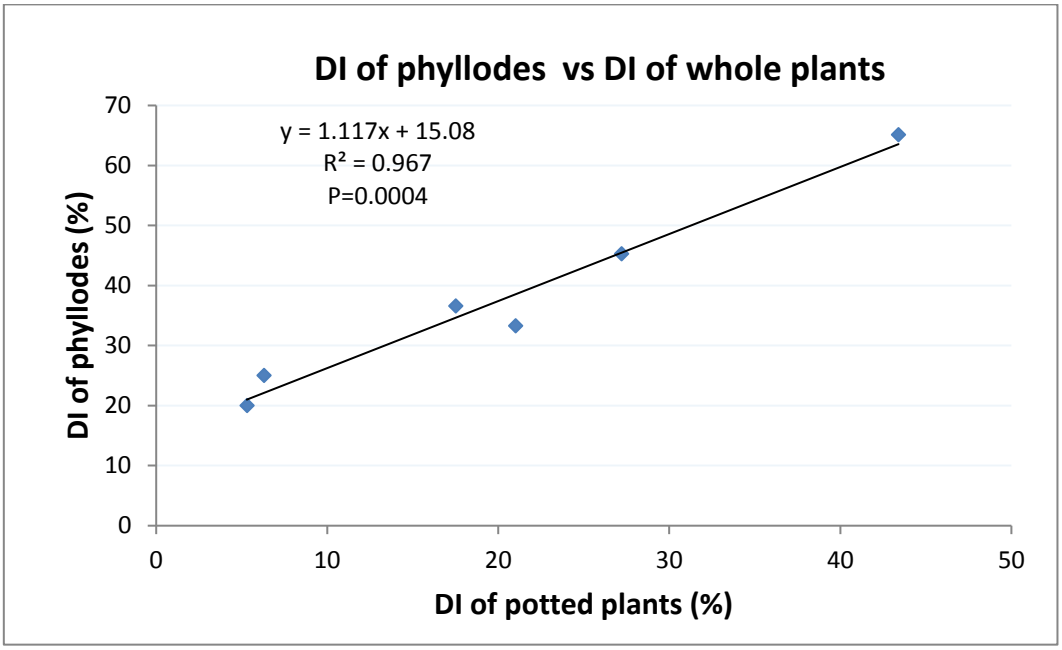


Figure III-9. Regression analysis among three screening protocols (phyllode, stem and potted inoculation) against three species of *Acacia* (*A. mangium*, *A. crassicarpa* and *A.* hybrid). Correlation between phyllode and potted plant inoculations and between detached stem segment and potted plant inoculations are significant.

Table III-5. Comparison of discolouration indices (DIs) of clones of *Acacia mangium*, *Acacia crassicarpa* and *Acacia* hybrid inoculated with *Ceratocystis manginecans* using different screening protocols. Data are expressed as mean values ± standard error (SE) of discolouration indices at two or five weeks after inoculation (WAI). Different letters (a-d) indicate statistically significant differences at a single assessment (LSD test, followed by ANOVA, $p<0.05$).

Potted plant inoculation protocol			**Phyllode inoculation protocol**			**Stem segment inoculation protocol**		
Species	**Clone**	**Mean value internal DI (%) 5 WAI**	**Clone**	**Isolate**	**Mean value DI (%) 2 WAI**	**Clone**	**Isolate**	**Mean value internal DI (%) 2 WAI**
A. mangium	RPT00021	43.4 ± 1.6^{a}	*A. mangium*	RPT00021	65.1 ± 1.2^{a}	*A. mangium*	RPT00021	7.8 ± 0.5^{a}
A. mangium	RPT00316	27.2 ± 1.6^{b}	*A. mangium*	RPT00316	45.3 ± 1.2^{b}	*A. mangium*	RPT00316	6.1 ± 0.5^{b}
A. hybrid	RPX80537	21.0 ± 1.6^{c}	*A.* hybrid	RPX80498	36.6 ± 1.2^{c}	*A.* hybrid	RPX80498	5.3 ± 0.5^{bc}
A. hybrid	RPX80498	17.5 ± 1.6^{c}	*A.* hybrid	RPX80537	33.3 ± 1.2^{c}	*A.* hybrid	RPX80537	4.5 ± 0.5^{cd}
A. crassicarpa	RPT30020	6.3 ± 1.6^{d}	*A. crassicarpa*	RPT30020	25.0 ± 1.2^{d}	*A. crassicarpa*	RPT30003	3.1 ± 0.5^{d}
A. crassicarpa	RPT30003	5.3 ± 1.6^{d}	*A. crassicarpa*	RPT30003	20.0 ± 1.2^{d}	*A. crassicarpa*	RPT30020	3.1 ± 0.5^{d}

III.4. Discussion

The potted plant inoculation technique showed the ability of *C. manginecans* to produce visible external lesions on the stems two weeks after inoculation and also demonstrated variation in tolerance among the five clones of *A. mangium* tested. This indicates the potential for genetic improvement by selecting more resistant or tolerant material for breeding programs. This procedure has been used in many studies to evaluate the pathogenicity of *Ceratocystis* isolates or resistance of plant species or genotypes, even though it has been argued that greenhouse experiments may yield different results to field experiments (Van der Westhuizen et al. 1992). However, compared with field inoculation tests, potted plant inoculation is more cost and time-efficient, avoids introducing more disease inoculum in the field and presents the potential for evaluating the pathogenicity of non-native isolates (Van Wyk et al. 2010). Field trials are an important component of breeding programs but a rapid, preliminary screening can expedite selection of more tolerant material.

Another important point which must be considered in procedures using potted plants is the length of time required. This procedure took approximately 18 weeks for screening one set of acacia clones, starting from seedling preparation, inoculation and obtaining results of relative tolerance and susceptibility. From both of our acacia potted plant inoculation tests, disease symptom or internal lesion could be recorded 5 weeks after seedlings were inoculated. At this time, the plants can be harvested, and the internal lesions assessed as most of the seedlings which were indicated to be highly susceptible to ceratocystis were wilting and phyllodes drying out, indicating that the maximum infection levels had been reached. While potted plant trials are faster than field trials, an even more rapid test would expedite the screening process when dealing with large numbers of clones. Given the low levels of

exploitable resistance in *A. mangium* (Brawner et al. 2015), it is anticipated that it will be necessary to screen a very large number of genotypes to establish a resistance breeding program.

C. manginecans isolates which were used in this study showed variability in aggressiveness. These fungi were isolated from the same tree species (*A. mangium*) but were obtained from different areas which may explain the variability in their pathogenicity. Previous studies of *Ceratocystis* aggressiveness have indicated that pathogenic variability may be due to genetic variation in the isolates which can be correlated with geographic area of origin and host species (Baker et al. 2003; Harrington et al. 2011). It is important to identify the aggressiveness of individual isolates, because pathogen variability is considered a crucial point for successful resistance breeding. The pathogen may experience a mutation due to their genotypic diversity which then leads to a break-down of the resistance gene in the breeding plant (McDonald & Linde 2002).

The inoculum form, i.e. spore suspension or mycelial plug, only produced a difference in aggressiveness in the early stages of infection, with higher disease measures from mycelial inoculum. At subsequent assessment dates, the DI showed no difference between mycelial plug and spore suspension. In general, both inoculum forms contain ascospores, conidia, chlamydospores and mycelia or mycelial fragments. The difference of lesion length in the earlier infection may be due to an immediate start of the infection process by mycelium while spores in suspension need to germinate before starting to infect the plant tissues. However, the important point in obtaining successful infection and identification of resistant/tolerant genotypes is more likely careful selection of isolates for aggressiveness and optimisation of inoculum concentration. The mycelium plug size and spore suspension concentration in this study were shown to be adequate for producing stem lesions. This is consistent with a previous study of *Ceratocystis* inoculation which showed that between 10^4 to 10^5 spores is an appropriate

concentration for disease incidence (Sanches et al. 2008). The results of the current study indicate that both inoculum forms can be used in pathogenicity tests with *C. manginecans*.

The *A. crassicarpa* clones used in this study were demonstrably more tolerant to *C. manginecans* infection than *A. mangium*, with much smaller lesions, and in some *A. crassicarpa* clones, a complete absence of external lesions on the inoculated stems. The results in this study are similar to those of Tarigan et al. (2013), where *A. crassicarpa* and *A. auriculiformis* were more tolerant compared with *A. mangium.* Thus, both species are considered as promising sources of resistance against ceratocystis wilt and canker disease in the breeding program.

It was difficult to avoid desiccation and contamination in the stem segment inoculation experiments, shortening the time available to assess the lesions on the stem segments. In this study, the cut stems were only cleaned using sterilized cotton prior to inoculation and incubation. A more robust surface sterilization may improve the potential for rapid screening using stem inoculation. Furthermore, low water availability in stem segments, particularly in the phloem and cambium, due to evaporation may affect the spore germination or mycelial growth in the stem. Phloem and cambium usually are used by *Ceratocystis* in the penetration and colonization phase (Zalasky 1965). This can prevent successful *Ceratocystis* development and infection of xylem tissue (Zalasky 1965; Keane & Kerr 1997), resulting in shorter lesions in stem segments.

Preliminary tests to prevent the desiccation using wax, moistened cotton or sand has shown that wax was more effective to control water evaporation. However, it may maintain sufficient moisture for a maximum of two weeks in stem size used here, due to evaporation through the entire stem segment surface. The size of stem segments also may have contributed to increased desiccation. In this experiment, we inoculated stem segments of less than 3 cm diameter. Larger stem segments, with a lower surface are: volume ratio, may delay desiccation,

providing adequate time to assay the lesions on the stem segments. A previous inoculation study using 40 cm long bolts from six year old *E. grandis* successfully avoided desiccation and showed significant correlation with field inoculations (Van Wyk et al. 2010). Our experiment however, showed that this protocol could discriminate the response among clones as well as species against *Ceratocystis* infection, even though finer discrimination between the highly susceptible *A. mangium* clones was not as clear as in potted plant and phyllode inoculation procedures.

The phyllode inoculation assay took far less time and effort to establish than the pot trials and needed only 2-3 weeks to complete. This procedure could detect the differences of susceptibility among clones of *A. mangium* as well as three tested *Acacia* species. It also showed a correlation with potted plant inoculation by reflecting an almost identical trend of disease incidence (lesion and necrosis) on stems and phyllodes. This study agrees with previous research by Magalhães et al. (2016) which showed the significant correlation between lesion width on cacao stem and leaf assays after inoculating with *C. cacaofunesta*. Newhouse et al. (2014) also found a similar result, where patterns of lesion and necrosis on chestnut stem and leaf were relatively similar after inoculating with *Cryphonectria parasitica*. Therefore, this protocol is considered to be a suitable indirect method for preliminary screening of plants for genetic resistance.

The phyllodes assay may be particularly useful to screen larges number of acacia clones due to the rapidity of this screening process. Our study showed that the time required for testing of potted plants was 18 weeks, while the phyllode protocol only needed 2-3 weeks. This indicates that phyllode inoculation may be six times more efficient than greenhouse plant inoculation. A similar conclusion in terms of time efficiency of leaf inoculation compared with greenhouse or field inoculation was also reported from previous experiments on chestnut leaf by Newhouse et al. (2014) and also leaf inoculation for resistance screening in cacao

(Magalhães et al. 2016). Furthermore, preliminary experiments indicated that the phyllodes could be inoculated up to 24 h after sampling. We recorded no difference in results between phyllodes inoculated immediately after collection and those inoculated the following day after overnight storage at 4 °C. Moreover, inoculating phyllodes in the laboratory may reduce the possibility of fungal inoculum spreading to the environment.

Comparing these three protocols has demonstrated that results from potted plant and phyllode assays for screening the resistance of *Acacia* clones had a high correlation. However, the stem segment procedure produced an inconsistent result and the protocol needs improvement. Compared with potted plant and stem segment assay, the phyllode assay is much more efficient in cost and effort, allowing screening of a greater number of *Acacia* clones. Also, this procedure is easier to maintain in laboratory conditions. Therefore, the phyllode inoculation protocol has high potential to be used as a rapid, preliminary screening assay for resistance of *Acacia* clones against ceratocystis wilt and canker disease.

IV. Diversity of Culturable Endophytic Bacteria from *Acacia mangium* Willd. in Sumatran Plantations

Abstract

Bacterial endophytes living in roots, stems and phyllodes of *A. mangium* in Indonesian plantations were cultured in order to gain an understanding of their diversity. Samples from trees between one and five years old were collected and their culturable endophytic bacteria were identified by sequence analysis of their 16S rDNA. In total, 278 bacterial isolates were derived from 270 samples representing 90 trees. The majority of bacteria were isolated from roots and more isolates were obtained from young acacia trees than from the older trees. Analysis of 16S rDNA sequences grouped the endophytic bacteria into five clusters; Firmicutes, Alphaproteobacteria, Betaproteobacteria, Gammaproteo-bacteria and Actinobacteria. Firmicutes were predominant with 66.2% of the isolates belonging to this group. Among 25 genera which were isolated successfully, *Bacillus* and *Burkholderia* were revealed as the most frequently isolated endophytic bacteria in *A. mangium*. Several of the genera isolated from *A. mangium* include species which have been previously demonstrated to have potential as biological control agents (BCAs) against plant pathogens as well as the ability to enhance plant growth. Further studies on the potential of these bacterial endophytes to assist growth and survival of *A. mangium* in Indonesian plantations are planned.

IV.1. Introduction

Diverse microorganisms colonize both external and internal plant tissues as their habitat (McInroy & Kloepper 1994). Microorganisms including fungi and bacteria which live in plant tissues internally with no negative impact on their host are known as endophytic organisms

(Schulz & Boyle 2006), though some authors argue that the term should apply to all organisms that spend all or part of their life cycle within plant tissues (Hardoim et al. 2015). Endophytic organisms may include archaea and unicellular eukaryotes as well as the more commonly studied fungi and bacteria (Hardoim et al. 2015).

Endophytes play an important role in plant health, promoting plant growth and protecting plants against unfavourable conditions such as flooding, drought, salt or heavy metals (Mercado-Blanco & JJ Lugtenberg 2014; Vandenkoornhuyse et al. 2015). In association with plants, endophytic bacteria can assist in the phytoremediation of contaminated environments such as soil and water contaminated by organic pollutants (Sessitsch et al. 2013; Afzal et al. 2014; Glick 2014; Ma et al. 2016). Potential benefits to plants include assistance in nutrient acquisition through nitrogen fixation, phosphate and potassium solubilisation as well as protection from pathogen infection and insect pests (Hallmann et al. 1997; James & Olivares 1998; Sturz et al. 2000; Bacon & Hinton 2006; Mercado-Blanco & JJ Lugtenberg 2014). Furthermore, some endophytic bacteria may also encourage their hosts to reduce production of the plant hormone ethylene which promotes tissue senescence. The bacteria then produce the enzyme *1-aminocyclopropane-1-carboxylic acid deaminase* which reduces the effect of ethylene, thus assisting the plants in response to environmental stresses such as drought, osmotic stress and soil salinity (Cheng et al. 2007; Glick et al. 2007).

Endophytic fungi or bacteria may colonize roots, stems as well as leaves intracellularly and/or extracellularly for all or part of their life cycle (Wilson 1995; Saikkonen et al. 1998). However, unlike endophytic fungi, endophytic bacteria colonize primarily intercellular and vascular tissue. Therefore, they might move within plant tissue through the xylem (Kobayashi & Palumbo 2000; Schulz & Boyle 2006). Endophytic bacteria grow more prolifically in roots of host plants compared to stems and leaves and naturally occurring wounds in roots are thought to be the main entry point for endophytic bacteria (Sprent & De Faria 1988). However,

their growth, diversity and total population in plant tissue may vary due to factors such as plant source, plant age, tissue type and environmental influences where the plants grow (Kobayashi & Palumbo 2000).

Associations with endophytic fungi and bacteria have been reported in a wide range of plants, from grasses and herbaceous crops to woody trees (Carroll & Carroll 1978; Clay 1988; McInroy & Kloepper 1995; James & Olivares 1998; Currie et al. 2019). Diversity of fungal and bacterial endophytes has been investigated in tropical and sub-tropical as well as temperate climatic zones, with greater abundance reported in tropical and sub-tropical plants (Banerjee 2011). Their occurrence and role in a range of tree species has also been investigated. For instance, abundance and diversity of endophytic fungi was greater in sapwood than in leaves of wild *Hevea brasiliensis* in Peru (Gazis & Chaverri 2010). Fungal endophytes were also isolated more frequently from surface-sterilised needles of *Pinus monticola* than from surface-sterilised seeds (Ganley & Newcombe 2006).

Over 100 isolates of endophytic fungi were obtained from *Theobroma cacao* and *Theobroma grandiflorum* in Brazil (Hanada et al. 2010). Meanwhile, several species of endophytic bacteria have been isolated from roots, stems, leaves, flowers and seeds of woody trees. Examples include *Bacillus alvei* from woody tissue of *Quercus* sp. (oak trees), *Bacillus thuringiensis* from roots and stems of *Gossypium hirsutum* (cotton), *Burkholderia cepacia* from roots and stems of *Citrus limon* (lemon), *Zea mays* (corn) and cotton, and *Enterobacter cloacae* from roots, stems, and fruit of *Vitis vinifera* (grape), *Cucumis sativus* (cucumber), corn, cotton and lemon (Kobayashi & Palumbo 2000; Lodewyckx et al. 2002).

Despite many reports of endophytic bacteria enhancing plant growth and acting as biological control agents by suppressing or inhibiting pathogen infection, these reports are mostly on agricultural crops and less information is available for woody plants or forest trees

(Kobayashi & Palumbo 2000). However, the studies of potential endophytic microorganisms in forest trees is currently thought to be important due to the long life cycle and large biomass of trees which may create more favourable habitats for endophytic bacteria compared with the annual life cycle and restricted volume of crop plants (Izumi 2011). Furthermore, due to the increase in demand of wood products as well as the emerging threats to sustainability of forest plantations, endophytic bacteria can be considered as a method to overcome these challenges by increasing tree health and timber yields (Anand et al. 2006; Izumi 2011).

Forest plantations have expanded in Southeast Asia during the last two decades as a result of increasing wood product demand. Acacias and eucalypts are very important as raw material for the pulp and paper industry. The area planted to *Acacia* species in particular has reached 1.2 Mha in Indonesia (Harwood & Nambiar 2014b). Monoculture plantings and introduction of alien species in forest plantation has led to emerging pest and disease problems. Vascular wilt disease of *Acacia* is one of the emerging diseases which has seriously affected *Acacia* plantations (Tarigan et al. 2011a). In order to maintain sustainability of wood product, the possibility of employing endophytic bacteria as biological control agents is one of the strategies under consideration for management of this disease (Tran et al. 2018). As a first step, a greater understanding of the diversity, identity and roles of bacterial endophytes in *A. mangium* is required as different species of endophytic bacteria may colonize different host plants (Rosenblueth & Martínez-Romero 2006; Izumi 2011).

Studies to date of bacterial endophytes in acacia are mainly limited to nitrogen-fixing root symbionts (Birnbaum et al. 2016), though an isolate of *Methylobacterium* has proven effective in increasing bio-remediation of arsenic by *Acacia farnesiana* (L.) Willd (Fterich et al. 2012; Alcántara-Martínez et al. 2018). Other studies have applied bacterial endophytes from other tree hosts to acacia; *Enterobacter* sp. strain MCR1.48, isolated from mangrove trees,

selected for its ability to fix nitrogen and solubilise phosphorus and introduced to *Acacia polyphylla*, increased the growth of this tree species (Castro et al. 2018).

A better understanding of the endophytic communities in trees is desirable to provide baseline studies prior to attempts to improve tree and ecosystem health by manipulation of the microbiome (Mishra et al. 2019). Studies into the diversity of bacterial endophytes in other forest trees include species such as *Populus* spp. and *Eucalyptus* spp. (Izumi 2011). Schmidt et al. (2018) investigated endophytic bacteria in roots and leaves of a hybrid poplar clone while Miguel et al. (2016) characterised the microbial diversity of an *Eucalyptus* hybrid leaf endophytes at different growth stages up to 18 months of age. These, however, represent two of only a few studies into endophyte diversity in forest trees and little is known about endophytic bacteria in *A. mangium*. This study aimed to investigate the diversity of endophytic bacteria from various tissues of *A. mangium* trees at different ages in Sumatran plantations, Indonesia.

IV.2. Materials and Methods

IV.2.1. *Acacia mangium* selection and sampling

Healthy, vigorous *A. mangium* trees were selected from five different areas separated by 15 to 25 km and from plantations of six different ages (<1, 1, 2, 3, 4 and 5 years old) in Riau Andalan Pulp and Paper's plantations in Riau province, Indonesia. In total, 90 trees were selected from these five areas; three trees for each age at each site. Roots, stems and phyllodes were collected from each of the selected *A. mangium* trees. Healthy roots of approximately 4 – 10 mm in diameter were cut for sampling. Stem samples of 4 to 8 cm width and 10 to 15 cm in height were taken from the bark into the wood at a height of approximately 1 metre (Figure IV-1). New green shoots as well as older, dark green phyllodes were collected. The samples

were put into plastic bags and kept on ice, transported to the laboratory and processed immediately after collecting.

(a) **(b)** (c)

Figure IV-1. Samples of phyllodes (a), stem (b) and root (c) from one-year old *Acacia mangium.*

IV.2.2. Plant tissue surface sterilization

The surfaces of plant samples were sterilized in order to avoid contamination by microorganisms which might grow on the exterior of plant tissues. Root, phyllode and stem samples were cut into approximately five cm lengths and washed in running tap water. This process then was continued by aseptic serial sterilization procedure in a laminar flow cabinet, following the protocol of Muzzamal et al. (2012). Samples were soaked in 70% ethanol for five min before immersion in 0.9 % sodium hypochlorite solution for 20 min followed by three rinses in sterilized distilled water to remove the sodium hypochlorite. Finally, the samples were immersed in 10% $NaHCO_3$ solution to inhibit fungal growth.

IV.2.3. Isolation of culturable endophytic bacteria

The sterilized roots, phyllodes and stems were divided into small fragments of approximately 5-10 mm before plating onto isolating medium. Two different culture media were used to enhance the number and variety of isolated endophytic bacteria; Luria Bertani

(LB) agar (tryptone 10 g/l, yeast extract 5 g/l, sodium chloride 5 g/l, agar 17 g/l) and nutrient agar (NA) medium (peptone 10 g/l, beef extract 10 g/l, sodium chloride 5 g/l and agar 17 g/l). Four small fragments from each sample were evenly distributed into Petri plates which were then incubated at 28 ^{0}C for 4 days. Bacterial colonies were selected on the basis of gross colony morphology and purified onto fresh agar culture media (NA and LB) as putative endophytic bacteria.

IV.2.4. Amplification and sequencing of the 16S rDNA gene

The isolated endophytic bacteria were grown in LB liquid medium (LB with no agar) and incubated for 48 h at 25 °C, shaken at 150 rpm, then 100 µl of bacterial culture were pipetted onto FTA™ cards (Whatman, Little Chalfont, UK). A small square of FTA card (1mm^2) was cut out and transferred to a 0.2 ml tube and washed with FTA purification reagent (Whatman, Little Chalfont, UK) and TE buffer (10mM Tris-Cl, 1 mM EDTA, pH 8), following the manufacturer's instructions, before amplification with the primers 27F and 1492R (Rotenberg et al. 2007). Each 25 µl PCR reaction contained 1 x Polymerase buffer (Bioline, AU), 2 mM $MgCl_2$, 0.2 mg/ml BSA, 0.2 mM each dNTP, 0.25 µM each primer and 1 U Mangotaq™ polymerase (Bioline, AU). The thermocycler (Applied Biosystems 2720, Foster City, CA, USA) was programmed as follows: 94 °C x 1 min, 35 cycles of (94 °C × 30 s, 55 °C × 90 s, and 72 ° C × 150 s), followed by 72°C × 5 min. An aliquot, 5 µl, was electrophoresed at 5 V/cm for 30 min on a 1.2% agarose gel which was then stained for 15 min in 1 × GelRed (Biotium, Fremont CA) and visualized on a transilluminator. PCR products were sent to Macrogen, South Korea, for sequencing 16S rDNA in both directions using the primers 27F and 1492R. Chromatograms were viewed and edited using Chromas software (Chromas Lite version 2.1.1 Technelysium Pty Ltd). Identification was derived from sequence searches of the SILVA database (Quast et al. 2013) and the National Centre for Biotechnology Information (NCBI).

IV.3. Results

IV.3.1. Abundance of endophytic bacteria from acacia roots, stems and phyllodes

In total 278 isolates of endophytic bacteria (EB) were isolated from *A. mangium*. EB were isolated most frequently from roots, followed by stems, with the lowest number isolated from phyllodes. Of the 278 EB isolates, 143 (51.4%) were obtained from *A. mangium* roots (Figure IV-2).

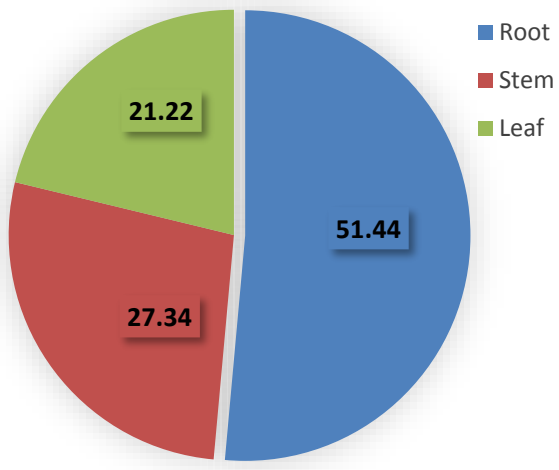


Figure IV-2. Percentage of total isolated endophytic bacteria from different parts of *Acacia mangium*; roots, stems and phyllodes.

IV.3.2. Abundance of endophytic bacteria in *Acacia mangium* trees of different ages

In general, EB were isolated more frequently from the youngest trees. This trend was clearest in root isolations (Figure IV-3a). For stem isolations, the highest numbers were obtained in one-year old trees whereas phyllode isolates peaked at four years of age (Figure IV-3b&c).

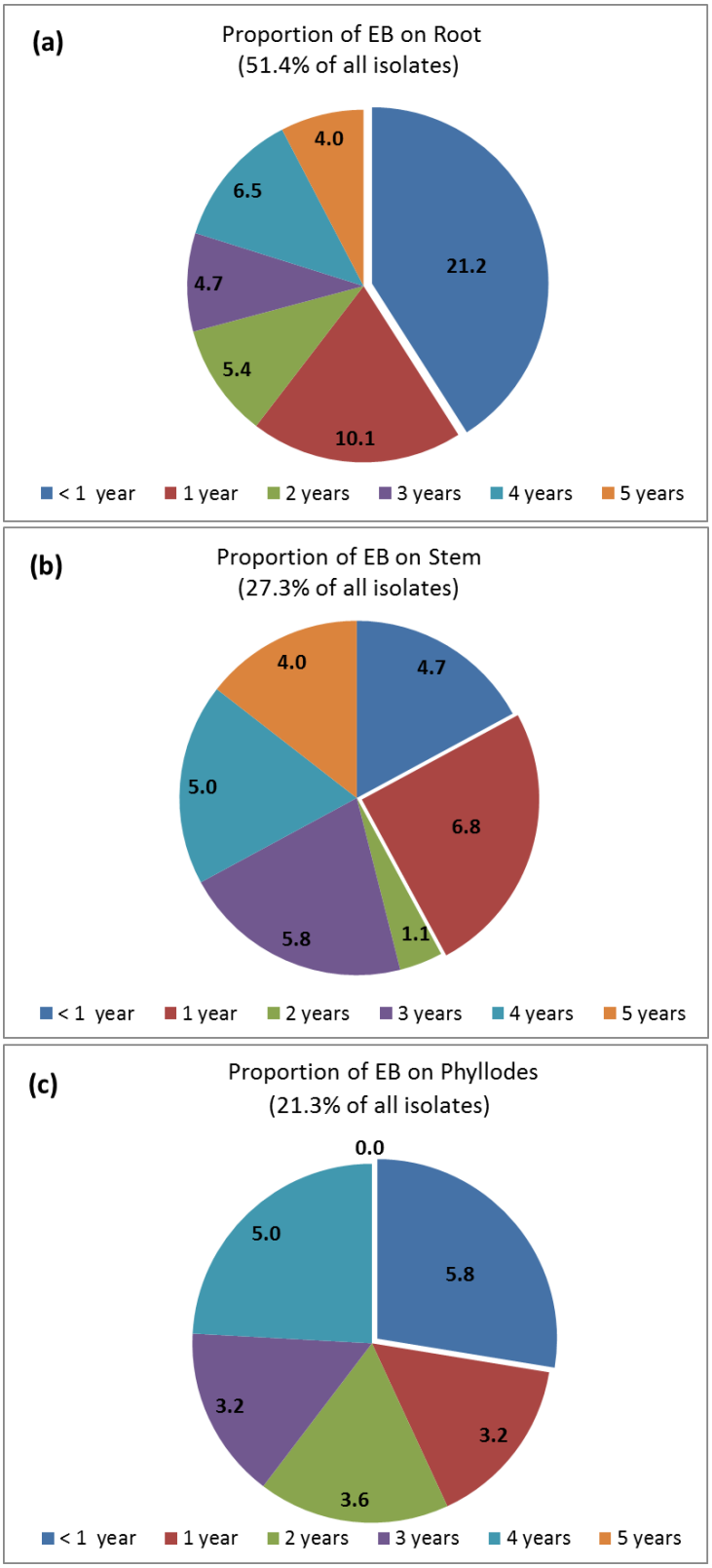


Figure IV-3. Percentage of endophytic bacterial isolates from different tree parts; (a) roots, (b) stems, and (c) phyllodes of *A. mangium* trees aged from <1 to 5 years old.

IV.3.3. Identification of isolated endophytic bacteria

Analysis of 16S rDNA sequence facilitated identification of 198 isolates to species or genus level (Table IV-1). These isolates belonged to five bacterial classes; Firmicutes, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. All five classes of bacteria were obtained from *A. mangium* phyllodes, though no Actinobacteria or Alphaproteobacteria were isolated from stems, nor were Actinobacteria isolated from root samples. The majority of bacterial isolates (more than 66%) belonged to the Firmicutes, while Betaproteobacteria and Gammaproteobacteria constituted 16.2% and 10.6% of EB isolates respectively. Even though Firmicutes bacteria predominated in number of isolates and number of species, however, the greatest number of genera (8 of 25 identified bacterial genera) was from the Gammaproteobacteria (Table IV-1).

The 131 isolates in class Firmicutes belonged to *Bacillus, Brevibacillus, Lactococcus, Lysinibacillus, Paenibacillus,* and *Staphylococcus*. These genera were abundant in *A. mangium* roots, stems and phyllodes, constituting 33%, 21%, and 11% respectively, of the isolates from these tissues. *Bacillus* was identified frequently from all tissues and was represented by 10 species. Three species of *Bacillus* - *Bacillus cereus, Bacillus thuringiensis* and an unidentified *Bacillus* sp., were the dominant species. Species isolated from a single tissue type, such as *Brevibacillus brevis* from roots, or *Lactococcus lactis* from phyllode were isolated rarely so tissue specificity cannot be inferred (Table IV-1).

The community of bacteria isolated from *A. mangium* roots was more complex than from stems or phyllodes. Almost all identified genera of bacteria in phyla Firmicutes, Betaproteobacteria and Gammaproteobacteria were found in roots. In contrast, no members of Alphaproteobacteria and very few of Actinobacteria were isolated from roots, almost all isolates from these groups were derived from phyllodes. Three species of bacteria; *Bacillus*

cereus, *Lysinibacillus* sp., and *Burkholderia* sp. were the most frequent root endophytes (Table IV-1).

The number of isolates from roots was higher than from stems and phyllodes, however, a greater variety of bacteria were found in phyllodes. The total of 25 genera of EB included 17 genera isolated from phyllodes, 15 genera from roots and 8 genera from stems. The genera such as *Methylobacterium, Asaia* and *Sphingomonas* were only isolated from phyllodes.

Table IV-1. Diversity of endophytic bacteria isolated from roots, stems and phyllodes of *Acacia mangium*, identified by sequencing of 16S rDNA.

Bacterial group	Identification of the isolates			Total	Total isolates from		
	Order	Genus	Species	isolates	Root	Stem	Leaf
Firmicutes	Bacillales	*Bacillus*	sp.	37	14	18	5
(131)*)			*cereus*	32	22	7	3
			thuringiensis	9	4	5	-
			subtilis	7	4	2	1
			pumilus	3	2	1	-
			altitudinis	2	-	2	-
			anthracis	1	-	-	1
			megaterium	1	-	1	-
			toyonensis	1	1	-	-
			pseudomycoides	1	-	1	-
		Lysinibacillus	sp.	9	7	2	-
			sphaericus	2	2	-	-
		Paenibacillus	sp.	4	4	-	-
			elqii	4	3	-	1
			alvei	1	1	-	-
		Staphylococcus	sp.	7	-	2	5
			epidermidis	3	-	2	1
			cohnii	1	-	-	1
			aureus	1	1	-	-
			warneri	1	-	-	1
			arlettae	2	-	-	2
		Brevibacillus	*brevis*	1	1	-	-
		Lactococcus	*lactis*	1	-	-	1
Total (%)				66.2	33.3	21.7	11.1
Betaproteobacteria	Burkholderiales	*Burkholderia*	sp.	17	13	2	2
(32)			*cepacia*	2	1	-	1
			cenocepacia	1	1	-	-
		Ralstonia	*mannitolilytica*	4	4	-	-
			sp.	3	3	-	-
			pickettii	1	1	-	-
			thomasii	1	1	-	-
		Cupriavidus	sp.	1	1	-	-
	Neisseriales	*Chromobacterium*	sp.	1	1	-	-
			subtsugae	1	1	-	-
Total (%)				16.2	13.6	1.0	1.5
Gammaproteobacteria	Enterobacteriales	*Enterobacter*	sp.	2	-	1	1
(21)			*aerogenes*	1	-	-	1
			asburiae	2	2	-	-
		Serratia	sp.	2	-	2	-
		Cedecea	*neteri*	2	-	1	1
		Klebsiella	sp.	4	1	2	1
		Pantoea	sp.	2	-	-	2
			agglomerans	1	-	-	1
	Xanthomonadales	*Dyella*	*marensis*	1	1	-	-
		Stenotrophomonas	sp.	1	-	-	1
	Pseudomonadales	*Pseudomonas*	*oryzihabitans*	1	1	-	-
			psychrotolerans	2	-	-	2
Total (%)				10.6	2.5	3.0	5.1

Table IV-1 Diversity of endophytic bacteria isolated from roots, stems and phyllodes of *Acacia mangium,* identified by sequencing of 16S rDNA (continued).

Bacterial group	Identification of the isolates			Total	Total isolates from		
	Order	Genus	Species	isolates	Root	Stem	Leaf
Actinobacteria	Micrococcales	*Brachybacterium*	sp.	2	-	-	2
(9)			*rhamnosum*	1	-	-	1
			paraconglomeratum	1	-	-	1
		Curtobacterium	sp.	2	-	-	2
			oceanosedimentum	1	1	-	-
		Micrococcus	sp.	1	-	-	1
		Ochrobactrum	sp.	1	1	-	-
Total (%)				4.5	1.0	0.0	3.5
Alphaproteobacteria	Rhizobiales	*Methylobacterium*	sp.	2	-	-	2
(5)			*komagatae*	1	-	-	1
	Rhodospirillales	*Asaia*	*bogorensis*	1	-	-	1
	Sphingomonadales	*Sphingomonas*	sp.	1	-	-	1
Total (%)				2.5	0.0	0.0	2.5

*) total numbers of EB isolates within group

IV.4. Discussion

The numbers of endophytic bacteria in plant tissues can be influenced by plant age as well as tissue type. In this study, higher numbers of EB were obtained from young *Acacia mangium,* in particular trees under one year old, and decreased gradually in older trees. This result agrees with a study on ginseng (Vendan et al. 2010) in which the highest abundance and diversity of EB occurred in plants less than 4 years old, and reduced drastically in mature plants. This can be explained by the fact that the plants attract the endophytic bacterial community by providing exudates or chemical compounds such as soluble sugars, proteins, amino acids, organic acids and other nutrients (Compant et al. 2005; Renaut et al. 2005). The different types and concentrations of these exudates correlate with the maturation stage of plants which then may impact the population and variety of endophytic bacteria in the plant tissues (Ferreira et al. 2008b).

Endophytic bacteria were obtained more abundantly from roots compared with stems and phyllodes. Diversity studies on other plant such as sugarcane (Mendes et al. 2007), ginseng

(*Panax ginseng*) (Vendan et al. 2010), Scots pine (*Pinus sylvestris*), silver birch (*Betula pendula*) and rowan (*Sorbus aucuparia*) (Izumi et al. 2008) revealed the same result to this study. Plant roots have been considered to be the main site for endophytic bacteria to enter plant tissues (Lodewyckx et al. 2002) through epidermal junctions, wounds and root hairs (Sprent & De Faria 1988). Furthermore, roots also produce exudates which contain various chemical compounds that attract and provide nutrients for endophytic bacteria (Lodewyckx et al. 2002).

Stems and leaves, however, have natural openings, stomata in leaves and lenticels in stems, through which potential endophytes can enter (Kluepfel 1993). Leaf and stem tissues also provide chemical compounds which serve as nutrients for endophytic bacteria, albeit of differing composition or concentration to root exudates (Lodewyckx et al. 2002). The aerial surfaces of plants or phyllosphere is lower in nutrients compared to the rhizosphere (Lindow & Brandl 2003). The phyllosphere environment is much more influenced by abiotic factors such as extreme fluctuations in temperature, moisture and radiation which may indirectly change the plant metabolism (Turner et al. 2013). Thus, this may explain the higher densities of endophytic bacteria in the roots compared with stem and leaf tissues, though phyllodes were inhabited by bacterial groups, Actinobacteria and Alphaproteobacteria, that were not isolated from roots.

The Firmicutes were predominant as endophytes in *A. mangium*. Among 25 genera in this group which were isolated successfully in this study, *Bacillus* was the genus with the widest distribution in roots, stems and phyllodes of *A. mangium*. This genus also was reported as dominant in other woody trees such as European deciduous and coniferous trees (Izumi et al. 2008), moso bamboo (*Phyllostachys edulis*) (Han et al. 2009) and *Eucalyptus* (Ferreira et al. 2008a). The ability of *Bacillus* spp. to dominate plant tissues may be due to their ability to produce antibiotic substances which inhibit other microorganisms as well as their ability to

survive in minimal nutrient concentrations in their environment (Lunares et al. 1993). These abilities also have been considered to enhance their potential as biocontrol agents (BCAs). Among the ten species of *Bacillus* which were obtained in this study, some species have been studied and demonstrated to have biological control potential. These include *B. subtilis* as an antifungal against wilt disease of banana (Souza et al. 2014), *B. thuringiensis* reported as an effective biopesticide (Ben-Dov 2014), *B. pumilus, B. megaterium* and *B. cereus* for the control of damping off and black stem disease of cucumber and tomato through induced systemic resistance mechanisms (Kloepper et al. 2004).

After *Bacillus* spp., *Burkholderia* was the second most frequently isolated genus from *A. mangium* roots. This genus is classed as Betaproteobacteria and may assist the plant to fix nitrogen (N_2) as has been reported for tropical legume plants (Moulin et al. 2001). Furthermore, *Burkholderia* also has potential to be used as BCAs due to their ability to produce metabolites which are effective antifungal agents, such as phenyl acetic acid, hydrocinnamic acid, 4-hydroxy-phenylacetic acid, and 4-hydroxyphenylacetate methyl ester (Mao et al. 2006).

Very few of the endophytic bacteria isolated in this study belong to the phylum Actinobacteria, and most of these were obtained from phyllodes. This can be explained by the very slow growth of Actinobacteria in culture medium compared with gram negative bacteria and fungal contaminants (Barka et al. 2016). Also, in this study, no specific medium was used in the isolation process. Specific media and treatments to the sample or medium which may inhibit gram negative bacteria and fungi are needed to enhance isolation of Actinobacteria (Goodfellow 2010).

Several genera of endophytic bacteria which were obtained in this study have the potential to be used as BCAs as well as enhance plant growth. Isolates of *Bacillus, Lysinibacillus, Paenibacillus, Burkholderia, Cupriavidus, Chromobacterium, Enterobacter,*

Serratia, and *Pseudomonas* all have potential to be used as BCAs (Kobayashi & Palumbo 2000; Compant et al. 2005). In addition, *Staphylococcus, Brevibacillus, Bacillus, Burkholderia, Methylobacterium, Micrococcus,* and *Sphingomonas* potentially have the ability to fix nitrogen and promote plant growth (Gray & Smith 2005; Ryan et al. 2008). Therefore, further studies of the benefits of these endophytic bacteria for *Acacia* trees could assist selection of candidates for inoculation programs to improve plantation productivity. Endophytic bacteria from other *Acacia* species, e.g. *A. crassicarpa*, which is less susceptible to *C. manginecans,* may also be worth exploring.

In conclusion, the diversity of endophytic bacteria in *Acacia mangium* in Indonesian plantations is most highly influenced by tree age as well as tissue type. Various genera of endophytic bacteria, predominately *Bacillus* and *Burkholderia*, were obtained from *A. mangium*, and their potential as BCAs and plant growth enhancers warrants further investigation.

V. Endophytic Bacteria Isolated from *Acacia mangium* Willd. in Indonesia as Potential Biological Control Agents of Ceratocystis Wilt and Canker Disease

Abstract

Endophytic bacteria isolated from *Acacia mangium* tissues were screened for their ability to inhibit growth of *Ceratocystis manginecans*, a fungal plant pathogen which causes wilt and canker disease in *A. mangium*. *In-vitro* assays testing 278 isolates of culturable endophytic bacteria resulted in 157 isolates which out-competed the pathogen, resulting in more than 70% growth inhibition when co-cultured. Nine isolates produced metabolites or putative antibiotic compounds that inhibited growth of *C. manginecans* in the absence of viable bacteria. The phylogenetic analysis of 16S rDNA from these nine isolates placed them in the genera *Paenibacillus, Lysinibacillus, Staphylococcus, Pantoea, Ralstonia, Cupriavidus* and *Ochrobactrum*. Several methods for inoculating *A. mangium* plants with these endophytic bacteria were evaluated. Applying liquid bacterial cultures to the root zone of germinating *A. mangium* seedlings was the most efficient inoculation method ($P<0.05$) when compared with inoculation by seed treatment, or by dipping or spraying of *A. mangium* stem cuttings.

V.1. Introduction

Endophytic bacteria are defined as bacteria that live internally in plant tissue without causing negative impacts on their hosts (Schulz & Boyle 2006). Some endophytic bacteria have demonstrated potential as biological control agents (BCAs) against plant disease (Wilson 1997; Pal & Gardener 2006). They have symbiotic, mutualistic and commensal relationships with their host plants (Ryan et al. 2008), and their potential to control plant diseases has been

explored in a range of plants including forest trees (Schulz & Boyle 2006; Izumi 2011). In agricultural crops, endophytic bacteria have been reported to successfully overcome not only pest and disease threats but also to enhance plant growth and to assist in phytoremediation of soil polluted with organic chemicals (Hallmann et al. 1997; Afzal et al. 2014) .

The role of endophytic bacteria as biological control agents against diseases of woody plants, particularly forest trees, has also been explored. For instance, Ren et al. (2013) demonstrated that *Bacillus pumilus* JK-SX001 effectively reduced poplar canker disease incidence by producing lytic enzymes including cellulases and proteases. Brooks et al. (1994) reported that endophytic bacteria in the genera *Bacillus* and *Pseudomonas* exhibited chitinase activity in an *in-vitro* inhibition assay and have the potential to reduce oak wilt disease if injected into *Quercus fusiformis* (oak trees) prior to infection by *Bretziella fagacearum*. Endophytic bacteria also may play an important role in accelerating development of resistant plants since they can elicit induced systemic resistance (ISR) which stimulates plant defence mechanisms against pathogens (Kloepper & Ryu 2006). In woody plants, the ability of endophytic bacteria to elicit ISR is considered as a more workable pathway to control plant pathogens such as vascular wilt disease instead of direct contact through antibiosis or production of secondary metabolites (Percival 2001), due to the slow movement of endophytic bacteria in plant tissues which is a potential problem as contact with the pathogen may be limited in an organism as large as a tree (Hallmann et al. 1997). This approach may also complement existing breeding and biotechnology programs for plant resistance (Percival 2001). However, reports of research describing the potential of endophytic bacteria to control diseases of forest trees currently are less numerous compared to agricultural crops.

The genus *Ceratocystis* includes several important fungal pathogens which cause serious disease and significantly reduce the productivity of many crops. This genus of fungi commonly causes rot diseases of agricultural crops, while in woody plants *Ceratocystis* spp.

cause canker stain and wilt disease (Roux & Wingfield 2009; Harrington 2013). In forest trees, *Ceratocystis* species have emerged as serious problems and caused significant mortality in plants such as *Quercus* spp. (oak trees) in USA (Juzwik 2007), *Eucalyptus* species in Brazil (Ferreira et al. 2011), Uruguay (Barnes et al. 2003a), and Republic of Congo (Roux et al. 2000a), *A. mearnsii* in eastern Africa (Uganda, Kenya) and southern Africa (Tanzania, South Africa) (Roux & Wingfield 2009), *A. mangium* in Indonesia (Tarigan et al. 2011a), Malaysia (Brawner et al. 2015), and Vietnam (Thu et al. 2012).

Attempts to overcome canker stain and wilt diseases caused by *Ceratocystis* species have focussed on the development of resistant planting material (Kile 1993; Roux et al. 1999; Harrington 2013). Selection of resistant or tolerant plants is a time-consuming process and the heritable variation in breeding process may become a challenge in this strategy (Brawner et al. 2015). In addition, genetic resistance may be short-lived when a pathogen evolves to overcome host defences (Maramorosch & Loebenstein 2009; Poland et al. 2009). In Brazil, where *C. fimbriata* causes a wilt and canker disease in eucalypts, resistant eucalypt clones have a life of around 10 years before succumbing to new genotypes of the pathogen (Oliveira et al. 2015). In *A. mangium*, only a low level of tolerance has been observed though selected hybrid clones of *A. mangium* and *A. auriculiformis* may be more tolerant (Trang et al. 2018). A combined approach based on genetic tolerance and augmented by biological control agents which may potentially inhibit the fungal pathogen or elicit ISR in plants may be more effective to protect acacia trees against ceratocystis wilt and canker disease. More information in terms of potential endophytic bacteria that inhibit *Ceratocystis* growth or trigger ISR as well as methods to introduce them into host tissues are important.

This study was designed to screen and evaluate *in vitro* the antagonistic ability of several endophytic bacterial strains, isolated from healthy, vigorous *A. mangium* in Indonesian forest plantations, against *C. manginecans*, a fungal pathogen which causes ceratocystis wilt

and canker disease in *A. mangium*. Methods to inoculate *Acacia* seedlings or cuttings with these endophytic bacteria in the nursery were also compared.

V.2. Materials and Methods

V.2.1. Isolate of *Ceratocystis* and endophytic bacteria

A single isolate of *C. manginecans,* CTA-138*, was used in this experiment. This fungus was selected from RAPP's culture collection on the basis of its demonstrated pathogenicity and robust identification by multilocus DNA sequencing (initially identified as *C. acaciivora* under code CMW 22595 (Tarigan et al. 2011a), since reduced to synonymy with *C. manginecans,* belonging to the *C. fimbriata* species complex (Fourie et al. 2015; Harrington et al. 2015). After seven days' growth on PDA 5 mm inoculum plugs for *in vitro* tests were taken from the growing edge.

Endophytic bacteria (EBs) were isolated from healthy, vigorous *A. mangium* trees in Sumatran plantations managed by RAPP (Chapter IV). A total of 278 endophytic bacterial isolates were screened *in vitro* for their antagonistic ability against *C. manginecans* (Table IV-1).

V.2.2. In-vitro assay

In-vitro screening experiments were separated into two procedures, dual culture and double streaks (non-contact) experiments following the methods of Zhou et al. (2014).

V.2.2.1. Dual culture test

Each EB isolate was multiplied in 100 ml nutrient broth medium (peptone 10 g/l, beef extract 10 g/l, and sodium chloride 5 g/l) in a 250 ml flask. The inoculated flasks were placed in an incubator shaker maintained at 28 °C, 150 rpm for 48 h. The number of cells per ml of

EB then was measured by spectrophotometry and then the suspension was diluted with sterile distilled water to obtain 10^8 CFU/ml. A 100 µl aliquot of each putative EB suspension was spread with a sterile glass spatula onto PDA medium in 90 mm Petri plates. A 5 mm diameter plug of *C. manginecans* culture which was grown on PDA medium for 7 days, was then placed in the centre of the Petri dish with the mycelium resting on the medium. A *C. manginecans* plug on PDA medium without putative EB was used as the control. Each treatment was replicated five times. The cultures were thereafter incubated at 24 ± 1°C with a photoperiod of 12 h dark and 12 h light. The radii of fungal growth were measured after 24, 48, 72, 96 and 120 h of incubation. The inhibition proportions were calculated daily by using the following formula:

$$I = \frac{C - T}{C} \times 100$$

(I is inhibition proportion, C is radius of *Ceratocystis* in control and T is radius of *Ceratocystis* in dual culture).

V.2.2.2. Non-contact test

Each EB isolate was inoculated in two parallel streaks 30 mm from opposite sides and 30 mm apart on a 90 mm Petri plate containing PDA medium and incubated for 24 h. Then, the 5 mm diameter inoculum plug of *C. manginecans* was placed in the centre of the Petri dish which was sealed and incubated at 24 ± 1 °C. Five replicates were prepared for each EB isolate. The average radial growth of *C. manginecans* towards the bacterial streaks (R2, Figure V-1), and the average radial growth in the perpendicular direction (R1, Figure V-1) were measured at 24, 48, 72, 96 and 120 h.

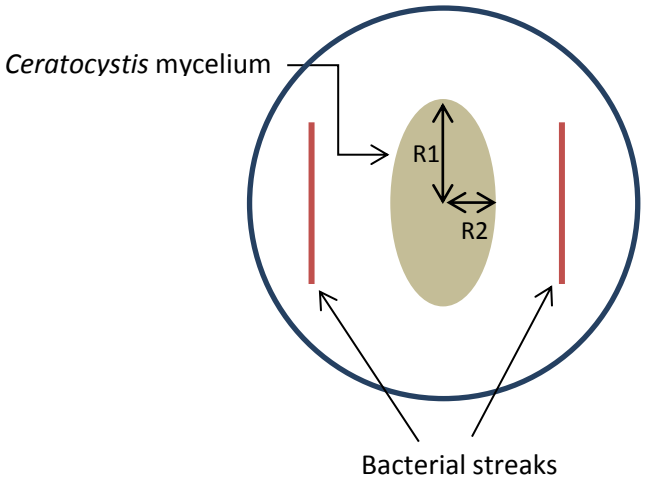


Figure V-1. Schematic illustration of non-contact *in vitro* inhibition test.

The percent of inhibition was calculated daily by following formula:

$$I = \frac{R1 - R2}{R1} \times 100$$

(I is inhibition proportion, R1 is average radial growth of *C. manginecans* parallel to the bacterial streaks and R2 is average radial growth of *Ceratocystis* towards the bacteria).

V.2.3. Metabolite test

The endophytic bacteria which showed the highest growth inhibition of *C. manginecans* in dual culture and non-contact tests were further tested for the presence of inhibitory metabolites. Two methods were tested; application of bacterial culture supernatant in agar wells and application of paper discs soaked in supernatant.

V.2.3.1. Agar wells method

Each endophytic bacterium was inoculated into 100 ml nutrient broth in a 250 ml flask and placed in an incubator shaker, set at 28 °C, 150 rpm, and incubated for 48 h. The bacteria were then centrifuged at 12879 g for 5 min. The supernatants were collected and used for

detecting secondary metabolites produced by EB isolates, following confirmation of sterility by plating onto LB medium.

Spore suspensions of *C. manginecans* were harvested from 7-days-old cultures grown on potatoes dextrose agar (PDA). The concentrations of conidial suspensions were counted using a haemocytometer and diluted with sterile distilled water to $2x10^5$ conidia per ml, and a 100 µl aliquot was spread onto each Petri plate using a glass spatula. This step was immediately followed by creating four wells with a diameter of 4 mm through the agar to the Petri plate bottom. The wells were created an equal distance apart on the Petri plate, and then 10 µl of supernatant from endophytic bacterial cultures was placed into each well. Each EB isolate was replicated in four Petri plates. Sterile distilled water replaced supernatant for control plates.

The growth of *C. manginecans* and the diameter of the clear zone around each well and were observed and recorded at 48, 96, 144 and 192 h after treatment.

V.2.3.2. Paper disc method

Supernatants from endophytic bacterial cultures and *C. manginecans* conidial suspensions were prepared as described above. Discs of 5 mm diameter were cut from Whatman filter papers (Ashless grade 41). Aliquots of *C. manginecans* conidial suspension, 100 µl, were spread onto PDA in Petri plates. The paper discs then were dipped into bacterial supernatant and four discs were placed in equal distance apart on the Petri plate. Four replicates were prepared for each endophytic bacterial isolate. For the control, paper discs were dipped into sterile distilled water.

The diameter of the clear zone around each paper disc was recorded at 48, 96, 144 and 192 h after treatment.

V.2.4. Amplification and sequencing of the 16S rDNA and *rpoB* genes

Endophytic bacteria which expressed the highest inhibition in dual culture methods as well as producing inhibitory metabolites in agar wells and paper disc tests were identified by DNA sequencing of the 16S rDNA. Aliquots of bacterial cultures were pipetted onto FTA™ cards from which a small square (1 mm^2) was cut out, transferred to a 0.2 ml tube and washed with FTA purification reagent and TE buffer before amplification with the primers 27F and 1492R (Rotenberg et al. 2007). Each 25 µl PCR contained 1 x Polymerase buffer (Bioline, AU), 2 mM $MgCl_2$, 0.2 mg/ml BSA, 0.2 mM each dNTP, 0.25 µM each primer and 1 U Mangotaq™ polymerase (Bioline, AU). The C1000 Touch ™ Thermal Cycler (BIO-RAD, UK) was programmed as follows: 94 °C x 1 min, 35 cycles of (94 °C x 30 s, 55 °C x 90 s, and 72 °C x 150 s), and 72 °C x 5 min. PCR products were electrophoresed on 1% agarose gels, stained with GelRed (Biotium, USA) and visualized on a trans-illuminator to confirm successful amplification before PCR products were sent to Macrogen, South Korea, for sequencing in both directions. Chromatograms were viewed and edited using Chromas software (Chromas Lite version 2.1.1 Technelysium Pty Ltd). Identification was derived from sequence searches of the SILVA database (Quast et al. 2013) and BLAST searches in the NCBI website (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was prepared by the Maximum Likelihood method using MEGA version 7.0 (https://megasoftware.net), analysed through Jukes-Cantor model and bootstrap on 1,000 replicates.

A fragment of the *rpoB* gene was amplified from target isolates (EB-05, EB-93 and EB-232) using primers *rpob1698f* and *rpoB2041r* (Dahllöf et al. 2000), and sent to Macrogen for sequencing. These sequences were aligned with *rpoB* sequences from related species available in GenBank and primers designed around variable regions (Table V-1).

Table V-1. Primers designed for detecting EB inoculated into *A. mangium* seedlings and cuttings.

Target isolate	*Primer name*	*Sequence*
EB05/EB93	EB05-F1	GCTTTATCGAAGCGCCTTATCGT
	EB05-R1	CGACTCGCTCTTTCGGAAGC
EB232	EB232-F1	CCTTATCGTCGTATTGACCAT
	EB232-R1	CGCAGCTGAAACTACTTGTTTC

V.2.5. Inoculating selected EBs into *Acacia mangium*

The most effective EBs in the *in vitro* tests were selected and inoculated into *Acacia mangium* in the greenhouse (nursery). Both seedlings and vegetatively propagated stem cuttings were inoculated as these are both standard propagation methods for *A. mangium*. Seeds of *A. mangium* (batch number FAM 7075) and clonal cuttings from the same seed source were used in this experiment. Three EB isolates which included the genera *Paenibacillus* (EB-05 and EB-93) and *Lysinibacillus* (EB-232) were used and cultured in Luria-Bertani (LB) broth medium for 48 h at 28 °C. These bacteria were then diluted with sterile distilled water to 10^6 CFU/ml before inoculating onto *A. mangium*.

The experiment was designed as completely randomized design (CRD). The 20 treatments included three EB isolates and one control with five different inoculation methodologies. The experimental unit consisted of a tray of 30 seedlings and three replicates were included for each treatment. A 10^6 CFU/ml suspension of each of the three EB isolates, EB-05 (T1), EB-93 (T2) and EB-232 (T3), was prepared and the control was sterile distilled water (T4). Based on standard operating procedure (SOP) of *A. mangium* propagation, *Acacia*

seeds are treated by covering in boiling water and left to soak overnight before sowing into cocopeat growth medium which was prepared by mixing 5 kg fertilizer (Osmocote®, slow release N-P-K, Indonesia) per m^3 cocopeat. Stem cuttings were prepared by cutting the *A. mangium* shoots approximately 10 cm long and with half leaf (phyllode) left. The tip of stem cuttings which were to be rooted into cocopeat growth medium were then dipped into rooting hormone (Rootone-F with a.i NAA (0.067%), IBA (0.057%), Indonesia) prior to be rooted in the greenhouse. In this experiment, five inoculation methods were prepared based on the SOP; **soaking** A. *mangium* seeds in boiling water for about an hour, then transferring into EB suspension for overnight soaking before sowing (D1); **pouring** 5 ml of EB suspension onto root system of *A. mangium* seedlings one week after germination (D2); **spraying** 5 ml of EB suspension onto *A. mangium* seedlings one week after germination (D3); **dipping** stem cutting tips of *A. mangium* into EB suspension for 5 min, followed by dipping into rooting hormone prior to be rooted into cocopeat growth medium (D4); **spraying** 5 ml of EB suspension onto all surfaces of *A. mangium* cuttings, including stem and half leaf immediately after cuttings were rooted into cocopeat medium (D5). Each seedling or cutting was grown in a plastic tube 3 x 3 x 10 cm size with cocopeat medium and placed in a plastic tray. All treated seedlings and cuttings were then grown and maintained for 5 weeks in the nursery which was protected from rain and mist-irrigated every 25 min for 60 s. The seedlings and cuttings were then transferred into an un-covered nursery and irrigated by an automated irrigation system every 60 min for 5 min. After 8 weeks, plants were tested for colonisation.

V.2.6. Detection of endophytic colonisation

All treated *A. mangium* seedlings and cuttings were sampled. From each set of 30 replicates of the treatments, five seedlings or cuttings were randomly selected for detecting EB colonisation. The seedlings or cuttings were removed from the seedling tube and the cocopeat

medium washed off in tap water. The plants were surface sterilized following Muzzamal et al. (2012); the seedlings or cuttings were soaked in 70% ethanol for five min before immersion in 0.9% sodium hypochlorite solution for 20 min followed by three rinses in sterilized distilled water to remove the sodium hypochlorite. Finally, the seedlings or cuttings were immersed in 10% $NaHCO_3$ solution to inhibit fungal growth. The sterilized seedlings or cuttings then were cultured in LB broth for 48 h at 28 °C to encourage growth of endophytic bacteria prior to attempting detection of the inoculated strains. Two procedures were used to grow and detect inoculated EBs from the seedlings and cuttings. In the **first procedure**, four plants from each treatment were assessed. All roots, stems and phyllodes of a single seedling or cutting were cut into pieces approximately 2 cm long and then mixed and incubated in a single 500 ml flask per plant, in 100 ml of LB medium. In the **second procedure**, one plant from each treatment was assessed. All roots, stems and phyllodes of single seedlings or cuttings were cut approximately 2 cm long and then roots, stems and phyllodes were cultured separately in different 250 ml flasks to identify which tissue type was colonised effectively by an endophytic bacterial isolate. Three replicate seedlings or cuttings from each treatment were cultured. The bacterial cultures were then centrifuged and DNA extracted from the pellet using a modified CTAB protocol (William et al. 2012). Extracted DNA was amplified using primers designed from the 16S sequences of the EB isolates (Table V-1). A total of 240 PCR reactions were processed for procedure 1 (4 isolates including control x 5 inoculation methods x 4 seedlings/cuttings x 3 replicates), and a total of 180 PCR reactions for procedure 2 (4 isolates including control x 5 inoculation methods x 3 part of seedling/cutting (root, stem and phyllode) x 3 replicates). The thermocycler was programmed as follows: 94 °C x 1 min, 35 cycles of (94 °C x 30 s, 55 °C x 90 s, and 72 °C x 150 s), and 72 °C x 5 min. PCR products were electrophoresed on 1% agarose gels and plants scored for presence or absence of target genera. Presence/absence of a target

EB was then analysed using the general linear model (GLM) procedure of SAS (SAS system v 9.4; SAS Institute Inc., Cary, NC, USA).

As the primers used in the PCR assay were not isolate- or even species-specific, the PCR tests only indicate the potential presence of the target bacterium or a related species and statistical tests were used to confirm the higher incidence of PCR positives in the inoculated plants vs the uninoculated controls. The proportion of PCR positives under different treatment combinations was angular transformed (arcsin of the square root) before analysis using the general linear model (GLM) procedure of SAS (SAS system v 9.4; SAS Institute Inc., Cary, NC, USA).

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V.3. Results

V.3.1. Inhibition of *Ceratocystis manginecans* in dual culture

More than 50% of endophytic bacterial isolates significantly inhibited growth of *Ceratocystis manginecans*, with over 70% inhibition by 157 of the 278 isolates (Appendix 1). Several isolates, including EB-05 and EB-93, completely inhibited the growth of the fungal pathogen in dual cultures (Figure V-2 a&b). In contrast, only three EB isolates inhibited *Ceratocystis* growth more than 70% at 120 h when the non-contact protocol was followed (Appendix 1 and Figure V-2 c&d).

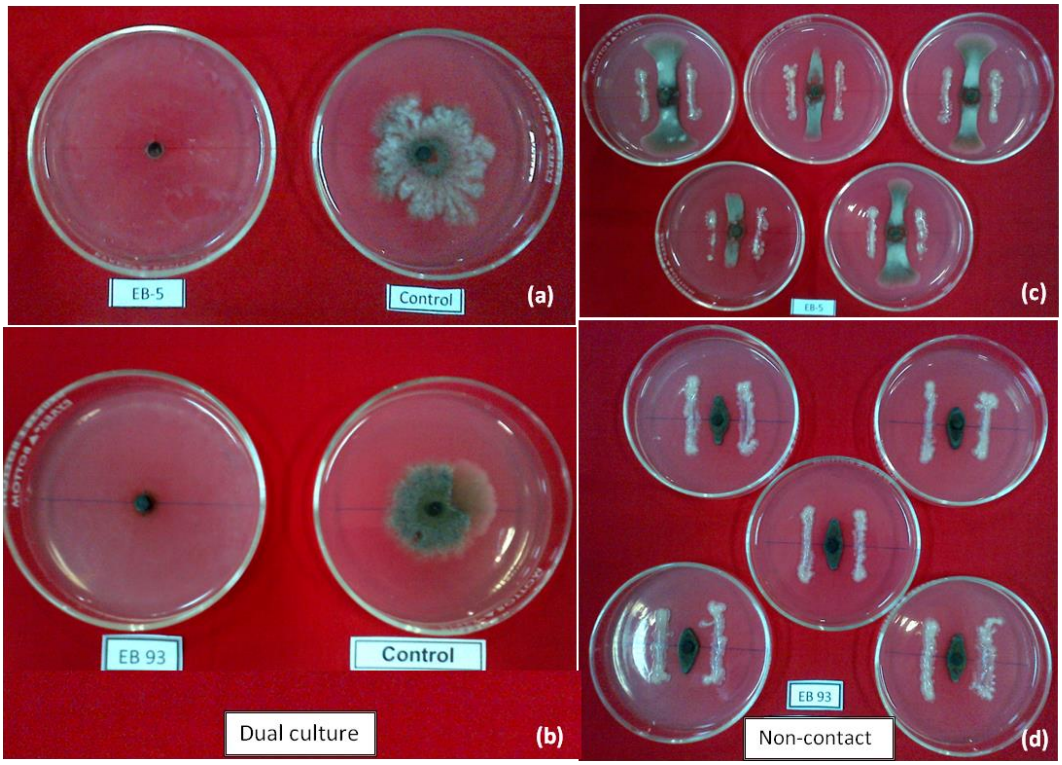

Figure V-2. Growth inhibition of *C. manginecans* by over 70% in dual cultures; contact procedure with bacterial isolate EB-05 (a), bacterial isolate EB-93 (b) and for the non-contact procedure, bacterial isolate EB-05 (c), bacterial isolate EB-93 (d).

V.3.2. Tests for production of inhibitory compounds

Endophytic bacteria did not grow from the supernatants when added to Petri dishes in wells or paper discs, confirming that the supernatant was free of viable cells. The supernatant assay revealed that six isolates (EB-14, EB-72, EB 73, EB-92, EB-93 and EB 232) produced a clear zone around wells and paper discs where the growth of *C. manginecans* was inhibited. The clear zone indicated that these isolates produced diffusible metabolites which blocked *C. manginecans* growth (Figure V-3 a-j).

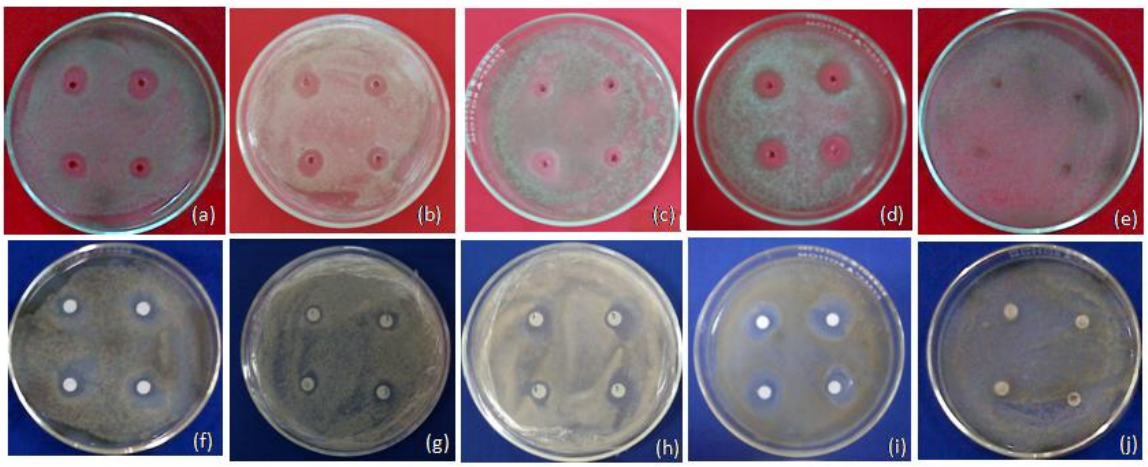

Figure V-3. Clear zones surrounding wells (a-e) or paper discs (f-j) demonstrate the inhibitory effect of metabolites in the supernatant of endophytic bacterial isolates at 120 h after inoculation with *C. manginecans* spores. Bacterial isolates; EB-72 (a,f), EB-73 (b,g), EB-92 (c,h) and EB-93 (d,i), controls (e,j).

Supernatant from the culture of endophytic bacterial isolate EB-93 produced the greatest clear zone in agar well and paper disc tests, with diameters of 4.6 and 4.1 mm, respectively. Even though the diameter of the clear zone tended to reduce over time, EB-93 had the greatest clear zone compared with other endophytic isolates from the first observation to 192 h after treatment (Figure V-4).

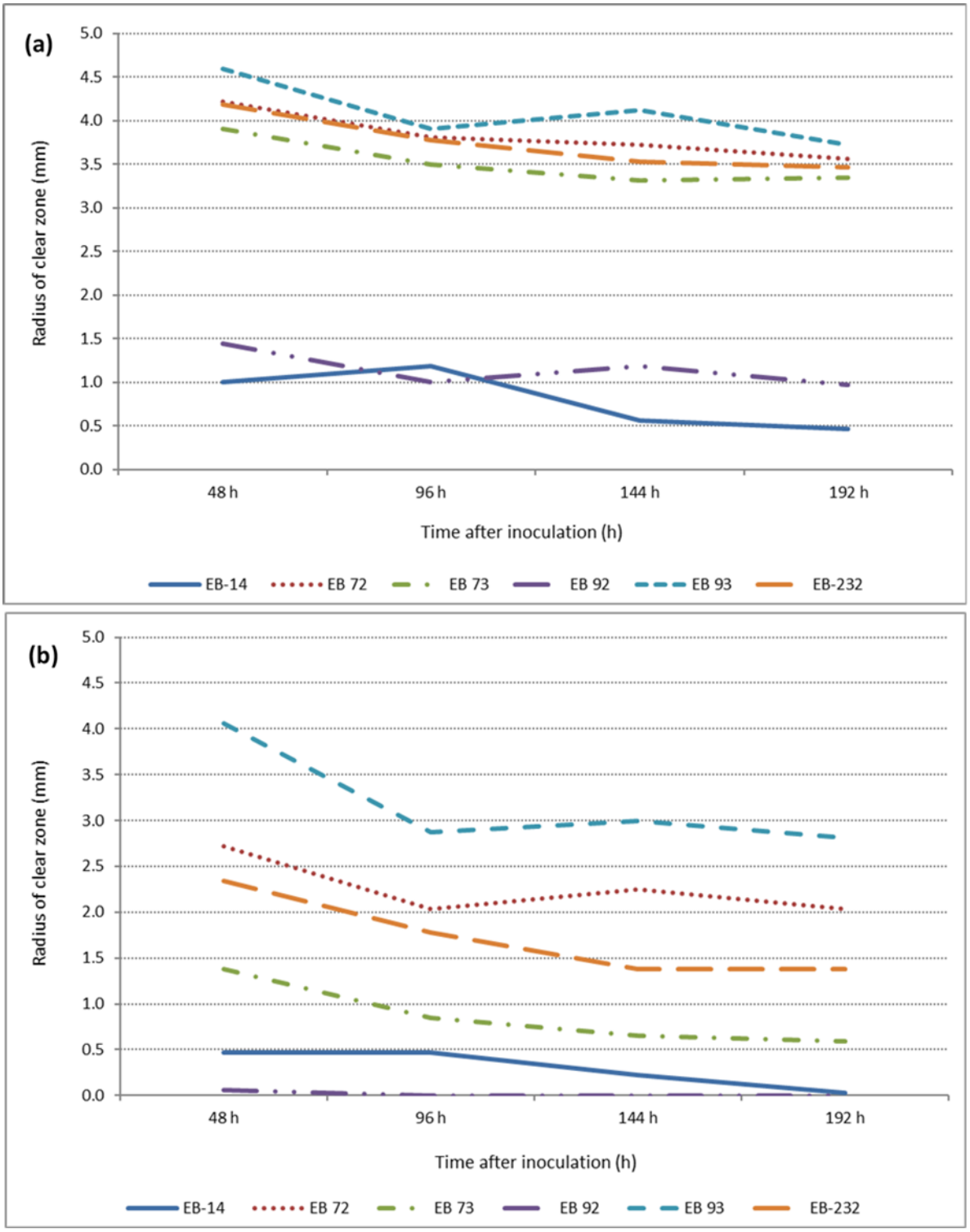

Figure V-4. Radius of clear zone surrounding wells or discs containing supernatants from cultures of six endophytic bacteria in agar media inoculated with *Ceratocystis manginecans* conidia after 48 to 192 h; (a) radius of clear zone using agar well method, and (b) radius of clear zone on paper disc assay.

V.3.3. Endophytic bacterial identification

Analysis of the 16S rDNA sequences from nine isolates of endophytic bacteria revealed that five promising isolates (EB-05, EB-06, EB-92, EB-93 and EB-232) belong to the phylum Firmicutes (Figure V-5). The nearest genera based on BLAST searches of the NCBI database were *Paenibacillus* for EB-05, EB-06 and EB-93, *Staphylococcus* for EB-92 and *Lysinibacillus* for isolate EB-232. Isolates EB-33 and EB-72 belonged to class Betaproteobacteria and the nearest genera were *Cupriavidus* and *Ralstonia* respectively. Isolate EB-14 was identified as a *Pantoea* sp. (Gammaproteobacteria), whereas EB-73 belonged to Alphaproteobacteria and nearest to *Ochrobactrum* (Table V-2 and Figure V-5).

Table V-2. Identification of endophytic bacterial isolates which were most effective in inhibiting growth of *C. manginecans* in dual culture and were tested for inhibitory metabolite production in agar well and paper disc assays.

Isolate code	Identification*)	Phylum	Inhibiting ability In dual culture (%)**)		Radius of clear zone (mm) ***)	
			Contact	Non-contact	Well agar	Paper disc
EB-05	*Paenibacillus* sp. 1	Firmicutes	82.9	72.6	0.0	0.0
EB-06	*Paenibacillus* sp. 1	Firmicutes	77.2	70.2	0.0	0.0
EB-93	*Paenibacillus* sp. *cf. elgii*	Firmicutes	86.4	63.0	4.6	4.1
EB-92	*Staphylococcus* sp. *cf. aureus*	Firmicutes	82.7	4.9	1.4	0.1
EB-232	*Lysinibacillus* sp. *cf. sphaericus*	Firmicutes	83.4	7.4	4.2	2.3
EB-73	*Ochrobactrum* sp.	Alphaproteobacteria	83.1	51.7	3.9	1.4
EB-33	*Cupriavidus* sp.*cf. plantarum*	Betaproteobacteria	77.4	70.0	0.0	0.0
EB-72	*Ralstonia* sp.	Betaproteobacteria	82.7	10.1	4.2	2.7
EB-14	*Pantoea* sp.*cf. cypripedii*	Gammaproteobacteria	82.9	0.0	1.0	0.5

*) Based on 16S rDNA sequence similarity ≥ 97%

**) Assessing 120 h after inoculation

***) Assessing 192 h after inoculation

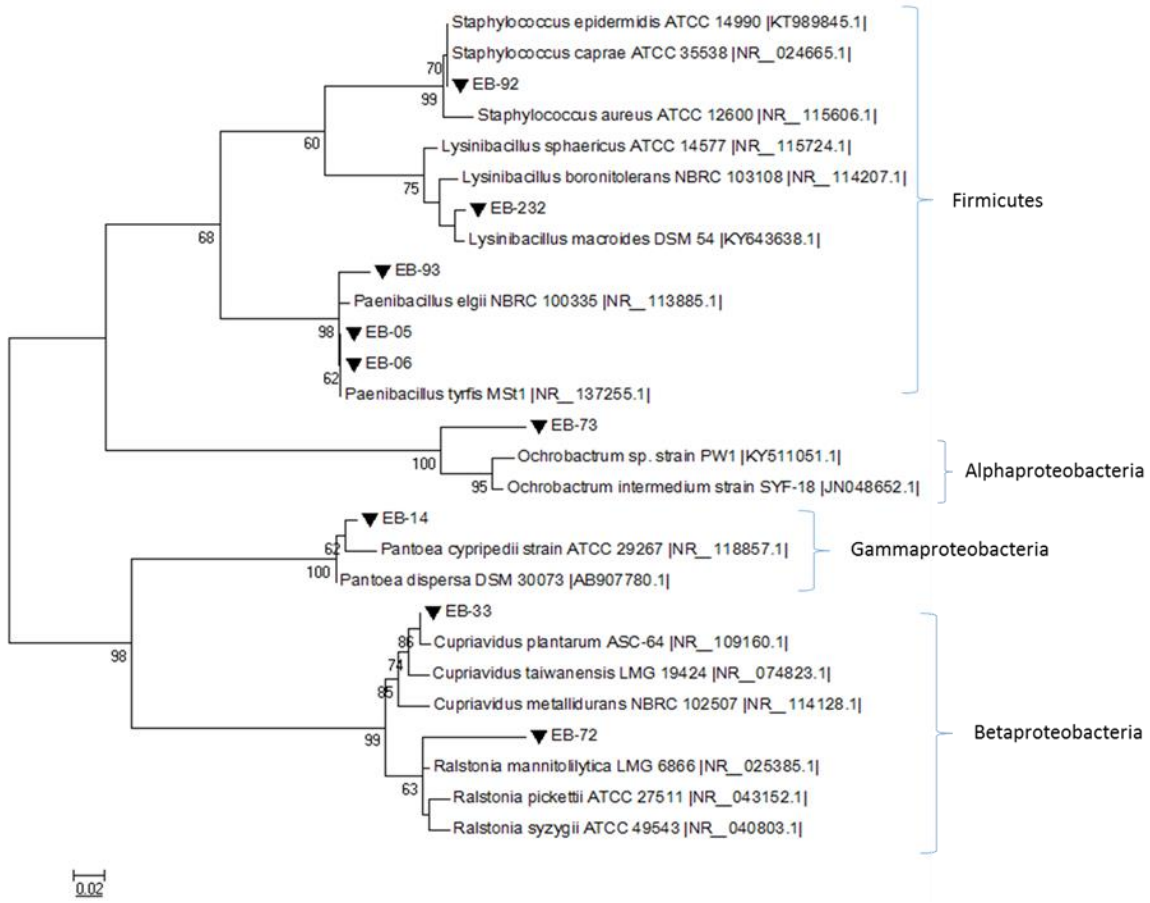

Figure V-5. Phylogenetic relationship among nine isolates of endophytic bacteria based on 16S rDNA sequences. The tree was built using Maximum Likelihood method and analysed through Jukes-Cantor model. Scale bar represents 2% expected variation, and numbers below branches indicate bootstrap values based on 1,000 replicates.

V.3.4. Inoculation of EB into *Acacia mangium*

All inoculation treatments of *A. mangium* gave positive PCR results, including un-inoculated controls, though significantly fewer of the control treatments were positive compared to inoculated plants (Figure V-6). The most effective methods of inoculating plants with EB were pouring a bacterial suspension over germinated seedlings in potting medium and spraying seedlings, with PCR positive rates of 91.7% and 80.6%, respectively. The pouring protocol was significantly better ($P<0.05$) at inoculating EB into *A. mangium* than pre-germination soaking of seeds, dipping or spraying of cuttings. It was not significantly different

from spraying EB-93 and EB-232 onto germinating *Acacia* seedlings, however, significantly different when spraying EB-05. The results were more consistent for all inoculated EB (91.7%) using the pouring method (Figure V-6a,b&c). The least effective method of inoculating bacterial endophytes was by spraying cuttings, with a positive detection rate of only 38.9%. Positive PCR results from non-inoculated plants indicated that these were also colonised by the target bacteria. However, the percentage detected was significantly lower than plants inoculated by even the least effective method.

All three inoculated EB isolates were detected in 91.7% of plants treated by pouring bacterial suspension onto germinating seedlings, with no significant differences among isolates. Colonisation rates varied slightly among the three bacterial isolates when alternative inoculation methods were employed. Higher colonisation rates by isolate EB-232 were detected in soaking and dipping methods compared with EB-05 and EB-93. However, when EB-232 was sprayed onto *A. mangium* stem cuttings, the detection rate was only 16.7%, significantly lower than EB-05 and EB-93, both of which recorded a 50% success rate, and no greater than uninoculated, control plants (Figure V-6c).

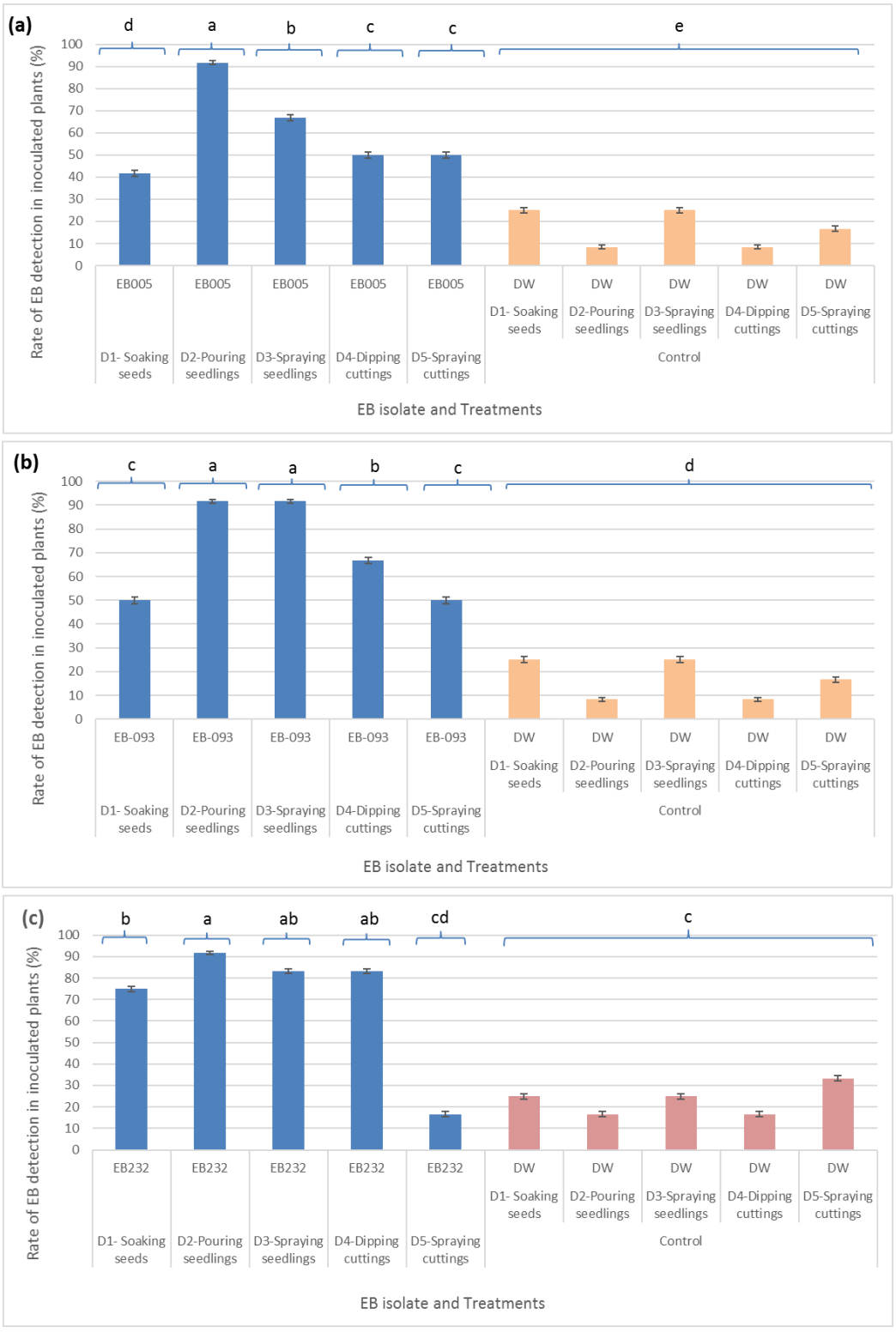

Figure V-6. Percentage of *A. mangium* seedlings or cuttings that tested positive for EB species 8 weeks after inoculation (WAI) for each inoculation protocol. EB-05 (a) and EB-93 (b) detected with primer pair EB05F1/EB05R1 and EB-232 detected with primer pair EB232F1/EB232R1(c) DW are the control plants inoculated with sterile distilled water, tested with the same primers. Treatments sharing the same letter were not significantly ($P<0.05$) different.

In the separately cultured root, stem and phyllode samples, PCR positives were more common following the pouring and spraying treatments of germinating seedlings. There was no significant difference (P<0.05) between these procedures. Less than half of the *Acacia* cuttings sprayed with an EB were PCR positive. This experiment also revealed that a higher percentage of root samples tested positive for EB colonisation; 62% compared to stems 43% and phyllodes 30%, P=0.0026. The recovery was 25 to 100% for all delivery methods, with higher recoveries from seedlings following inoculations by pouring, dipping cuttings and spraying germinating seedlings. The recoveries reached 100% for both isolates EB-93 and EB-232 (Figure V-7). Fewer PCR positives were obtained from phyllodes and un-inoculated *A. mangium*. Some of the uninoculated controls also tested positive for presence of the target EBs, but at a lower rate than inoculated seedlings, P=0.0013 (Figure V-7).

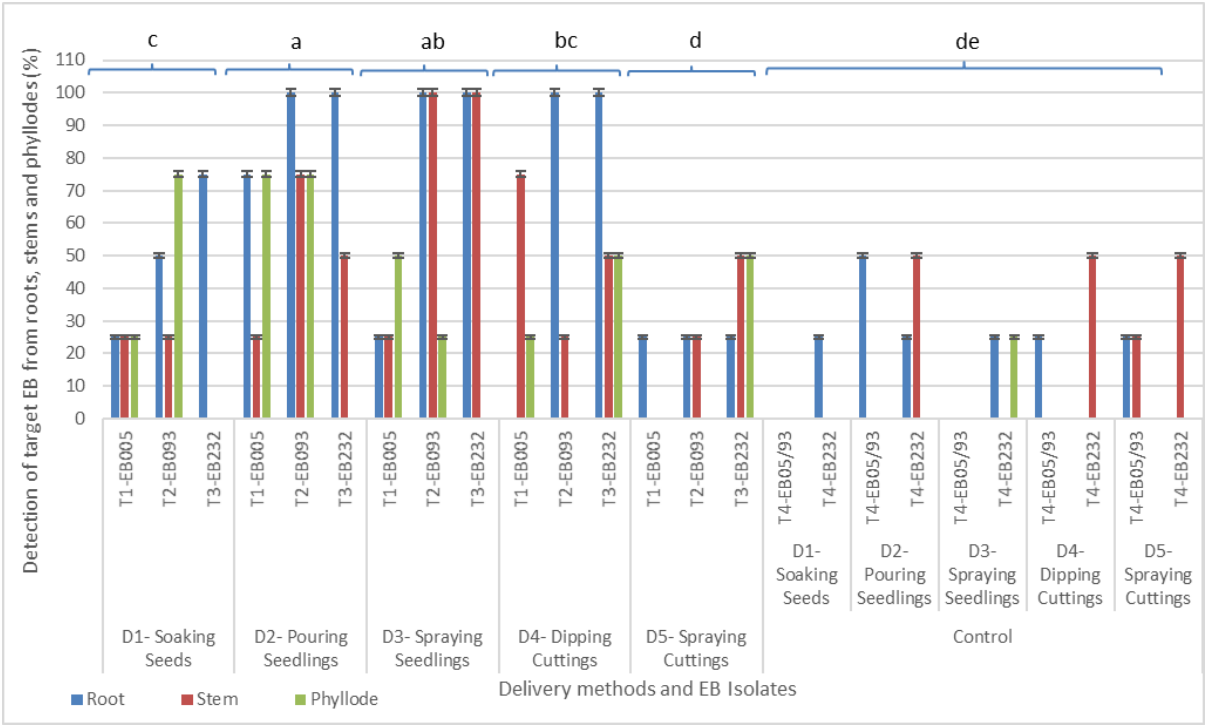

Figure V-7. Percentage detection of EB in roots, stems and phyllodes of *A. mangium* seedlings or cuttings for each inoculation protocol at 8 weeks after inoculation (WAI). T1, T2 and T3 refer to the isolates EB-005, EB-093 and EB-232, respectively, and T4 refers to inoculations with sterile distilled water (control). Percentages sharing the same letter were not significantly (P<0.05) different.

V.4. Discussion

The current study revealed that a high proportion of endophytic bacterial isolates from *A. mangium* had the ability to inhibit growth of *C. manginecans in-vitro*. Most of the bacterial isolates inhibited *C. manginecans* growth in *in-vitro* assay through resource competition. Relatively few isolates were shown to produce antimicrobial metabolites in tests using culture supernatants. The active components which were produced by endophytic bacterial isolates have not been identified. However, the clear zone between fungal growth and the supernatant from the culture of an endophytic bacterium indicated suppression of *C. manginecans* growth through an unknown mechanism of antibiosis. This mechanism has been considered as the main action of inhibition by endophytic bacteria given their known ability to produce various enzymes and antibiotics (Shali et al. 2010; Brader et al. 2014). In this study, the putative antibiotics were constitutively expressed as they were produced in liquid culture in the absence of *C. manginecans* and showed the ability to inhibit the growth of *C. manginecans*.

The endophytic bacteria which inhibited growth of C. *manginecans* were identified as belonging to six genera: *Paenibacillus, Staphylococcus, Lysinibacillus, Pantoea, Ralstonia* and *Ochrobactrum.* Among these genera, *Paenibacillus* and *Lysinibacillus* showed greater inhibition of *C. manginecans* growth. These genera belong to the Firmicutes, and are known to produce chitinolytic enzymes which are able to suppress various fungal plant pathogens (Williamson et al. 2000; Brzezinska et al. 2014). Moreover, *Paenibacillus* and *Lysinibacillus* can secrete several antimicrobial enzymes including glucanases, lipases, chitinases, cellulases and proteases which have a role in the decomposition of eukaryote cell walls (Grady et al. 2016; Naureen et al. 2017).

Growth inhibition of the target pathogen is a common feature of potential biological control agents, with previous studies confirming that antimicrobial compounds are often

enzymes associated with pathogenesis. For example, Singh et al. (2013) reported a *Lysinibacillus* sp. expressing strong antifungal activity against *Fusarium* spp. and *Macrophomina phaseolina* by production of chitinase. *Pantoea agglomerans* has also been reported to produce strong antifungal compounds and to effectively control onion bacterial streak and bulb rot disease caused by *Pseudomonas viridiflara* and *Xanthomonas* leaf blight caused by *Xanthomonas retroflexus* (Sadik et al. 2015). Lytic enzyme activity was expressed by *Ochrobactrum antrophi* and effectively suppressed blister blight disease caused by *Exobasidium vexans* on tea (Sowndhararajan et al. 2013).

In this study, growth inhibition by antifungal compounds from endophytic bacteria decreased over time. As growth inhibition is likely to be related to the concentration of the metabolite, pH and temperature (Zarei et al. 2011), it is postulated that potentially unstable metabolites decayed to some degree. While growth inhibition of the pathogen was demonstrated, it is not known if this mode-of-action would operate effectively to suppress disease development *in planta*. There is also the potential for pathogen populations to develop resistance to an antimicrobial metabolite (Morrissey & Osbourn 1999), similarly to fungal mechanisms of resistance against synthetic fungicides, where the gene mutations in the fungal pathogen may confer resistance to a particular active ingredient of fungicides (Deising et al. 2008). Therefore, validation in field trials is necessary and requires methods to inoculate host plants.

The two most successful methods for inoculating *Acacia* involved applying bacterial suspensions to germinating seedlings, either by pouring or spraying. Pouring bacterial inoculum onto seedlings a week after germination resulted in the highest detection of endophytic bacteria from the tested samples, with a 90% success rate for each of the three bacterial isolates. Spraying seedlings gave a lower success rate for two of the isolates though the difference was not significant. All other treatments had a significantly lower success rate,

with the exception of dipping cuttings into EB-232 inoculum. Dipping cuttings gave a higher colonisation rate than spraying cuttings with isolates EB-93 and EB-232 though this difference was not significant for EB-93. However, in this study application to germinating seedlings gave higher colonisation success than application to stem cuttings which indicated that endophytic bacteria are more likely to enter the *Acacia* tissues through root systems. This is also supported by the higher rate of detection in roots, followed by stems with the lowest detection in phyllodes, when plant parts were assessed separately. However, the high success rate for cuttings dipped into EB-232 inoculum indicates that there may be interspecific variation and methods need to be optimised for each bacterial isolate. The results also indicate that endophytic bacteria are more likely to enter the *Acacia* tissues through root systems. The endophytic bacteria which entered the root system may have colonised the root surface prior to entering acacia root tissues. During seed germination and root growth, wounds naturally occur in the germinating radicles or epidermal conjunctions, root hairs and secondary root emergence zone or sites of root branches which can be useful as the primary entry points of bacteria. These wounds not only create an entry point but also may provide a food source for the bacteria through leakage of plant exudates (Hallmann et al. 1997). This method of inoculating endophytic bacteria via roots agrees with results of previous studies. Algam et al. (2005) reported that introducing *Bacillus* through seed treatments and drenching resulted in abundant colonisation of endophytic *Bacillus* in tomato tissues compared with foliar spraying. Bressan and Borges (2004) also reported that endophytic bacteria were successfully introduced by pruning maize roots and treating with endophytic bacteria prior to planting.

This study also revealed that inoculation of endophytic bacteria onto germinating acacia seedlings had a higher success rate than *Acacia* stem cuttings. Endophytic bacteria colonising plant surfaces prior to tissue entry may be influenced by available food source, temperature, UV light and plant conditions (Hallmann et al. 1997). These responses may also be bacteria-

isolate specific (Hardoim et al. 2015). Unlike germinating *Acacia* seedlings, stem cuttings, while providing a wound as an entry point, do not supply a nutrient source during the lag phase before growth of roots and apices. In this situation, only endophytic bacteria adapted to this environment can survive, colonise the plant surface and then successfully enter the plant tissues (Hardoim et al. 2015).

In this study *Lysinibacillus* had a high success rate in colonising cuttings when exposed to the bacterial suspension for 5 min prior to planting but not when the suspension was sprayed onto cuttings after planting. Presumably this provided sufficient time for the bacteria to enter the vascular tissues via the wound in the tip of *Acacia* stem cuttings. Furthermore, when the treated cuttings were planted into growth media, the temperature, pH and oxygen available in the growth media may have enhanced the survival of endophytic bacteria on the surface of the stem cutting prior to colonisation of the growing root and entry to plant tissues (Frank et al. 2017). In contrast, spraying cuttings may have provided fewer entry points for endophytic bacteria. Factors potentially influencing bacterial numbers and colonisation in this situation include temperature and removal of bacteria by irrigation water applied during planting in the shade house.

The method used for detection of the three endophytic bacteria used in this study were not very specific, PCR positives could indicate the presence of the target or closely related species as primers were not rigorously tested for specificity. Even if this had been done, there would be no assurance that the positive detections were a result of inoculation as a wild strain could be present, as was most likely the case where control seedlings gave a positive result. In the current study, the PCR test results satisfied statistical criteria to support the efficacy of different inoculation methods. A more detailed study, and a higher level of confidence, would be possible if candidate BCA bacteria were transformed with a gene expressing a marker such as green fluorescent protein (Ferreira et al. 2008a).

Acacia mangium is usually propagated from seed but current research aimed at developing planting material that is resistant or tolerant to *Ceratocystis* is focussed on clonal hybrids because of the high susceptibility of *A. mangium* to *C. manginecans* (Brawner et al. 2015). It is unlikely that endophytic bacteria will be sufficient to control *Ceratocystis* disease in a highly susceptible species such as *A. mangium,* so, in the absence of other strategies for managing this devastating disease, it is likely that planting of *A. mangium* will not continue. The continuation of *Acacia* forestry in Indonesia is therefore dependent on the availability of hybrid planting material with a suitable degree of resistance or tolerance to *Ceratocystis.* In this case, deployment of endophytic bacteria with biological control activity against *C. manginecans* may be a useful supporting strategy and a method for inoculating cuttings will be required.

In conclusion, most endophytic bacteria isolated from *A. mangium* were able to inhibit the *in vitro* growth of *C. manginecans* significantly. Antibiosis of supernatants from *C. manginecans* cultures was demonstrated for six isolates belonging to Firmicutes, Alphaproteobacteria, Betaproteobacteria or Gammaproteobacteria. Inoculating *A. mangium* seedlings with endophytic bacteria, particularly through root drenching, was highly successful and can be considered the best method to deliver endophytic bacteria into *Acacia* seedlings. However, inoculation into cuttings may need to be pursued if *Acacia* clonal hybrid replaces, to some extent, *A. mangium* plantations in Indonesia, as has occurred in Vietnam (Nambiar & Harwood 2014).

VI. Symptoms and Signs of Ceratocystis Canker and Wilt Disease in *Acacia mangium*

Abstract

The symptoms and signs of ceratocystis wilt and canker disease in *Acacia mangium* were observed over a period of 19 weeks in Sumatra, Indonesia. Disease incidence nearly doubled over the short period of observation. The onset of the disease appeared associated in some cases with monkey damage or, more frequently in this study, with holes caused by pin borers. Following wounding, there was a rapid progression of stem discoloration, cracking, cankers, gummosis and the production of an alcoholic-smelling, frothy exudate. The final symptoms observed were yellowing phyllodes, wilting and finally death. However, before this stage the disease was associated with a reduction in leaf area index (LAI). This LAI data is potentially useful for the earlier detection of *Ceratocystis* incidence through aerial observation.

VI.1. Introduction

Acacia mangium is a fast growing tree species, able to fix nitrogen in the soil and shows tolerance to a wide range of soil and environmental conditions (Krisnawati et al. 2011). This species has been planted widely in Asian and Pacific countries for furniture and as a raw material of pulp and paper industries (Hegde et al. 2013). It has potentially high yields and quality particularly suited to producing high quality pulp and paper (Logan 1987) with recourse to fewer chemicals than other species during the process (Pinto et al. 2005). Therefore, this species has been planted extensively for pulpwood production, especially in SE-Asia over the last two decades.

Acacia mangium is, however, very susceptible to two diseases, root rot disease caused by the fungal pathogen *Ganoderma philippii* (Lee 2004; Mohammed et al. 2012; Francis et al. 2014), and ceratocystis wilt and canker caused by *Ceratocystis manginecans* (Tarigan et al. 2011a; Fourie et al. 2015). This latter fungal pathogen in particular has killed thousands of hectares of young acacia trees in Indonesia (Tarigan et al. 2011a) and Malaysia (Brawner et al. 2015). It is also a significant threat to forestry in Vietnam, where in 2011 it has caused 15-20% mortality of *A. mangium* and *Acacia* hybrid trees in plantations (Thu et al. 2012).

Symptoms of ceratocystis disease in woody trees, including *Acacia* trees, have been observed and reported by many researchers (Roux & Wingfield 2009). However, documenting the progression of ceratocystis disease symptoms and signs, particularly in *A. mangium*, is necessary for a better understanding of disease epidemiology to inform monitoring methodology and potentially to help assess disease management strategies. The aims of this study were to (a) monitor over time the progression of ceratocystis wilt and canker disease symptoms and signs in *A. mangium* from the first visible signs of infection, and (b) to investigate potential effects of this disease on the density of leaves in the canopy. The results of this study are discussed in relation to the potential for aerial remote sensing (Asner et al. 2003; Stone & Mohammed 2017) to detect changes in canopy density associated with the earlier stages of ceratocystis wilt and canker disease in *A. mangium*.

VI.2. Materials and Methods

VI.2.1. Observation plots

A 2-year old *Acacia mangium* plantation in Riau province, Indonesia, was used for monitoring the disease. The acacia trees were planted at a spacing of 3 x 2 m resulting in a density of 1,667 trees per ha. Two planting areas (I-028 and J-060) with ceratocystis disease

present were selected. Each area was 5 ha and approximately 5 km apart. Trees were approximately 14 m in height with a dbh of up to 11 cm. Ten trees in I-028 and five trees in J-060 were selected that were showing symptoms and signs associated with ceratocystis disease symptoms. For each of these trees eight, mostly asymptomatic neighbours were selected for monitoring i.e. a total of 135 trees were monitored. While the aim was to have a single infected tree surrounded by eight asymptomatic neighbours, this was not possible to achieve. Visibly infected trees were coded T1 – T15, and asymptomatic neighbouring trees around each infected tree were coded N1 – N8.

VI.2.2. Monitoring of ceratocystis wilt and canker disease

Trees to be monitored were assessed for the presence or absence of signs and symptoms usually associated with ceratocystis diseases in trees (Figure VI-1 and Figure VI-2) i.e. insect (borer) damage, with or without a white frothy exudate that is the product of fermentation by yeasts and bacteria; stem cracking (with and without gummosis (a thick, reddish gum produced by the tree)); stem discoloration; stem cankers (with or without gummosis); yellowing leaves; and wilting. Specific to this study, the incidence of bark stripping by monkeys was recorded.

Disease symptoms or signs on each tree were tracked and recorded every 2 weeks over a total of 19 weeks. At each assessment date the central tree in each plot was observed first, followed by its surrounding trees.

The data for six categories of symptoms or signs is presented i.e. ‘Monkey damage’, ‘Crack/Canker’, ‘Pin borers’, ‘ExLesion’ (external lesion or discoloration), ‘Yellowing leaves’, and ‘Wilting’. The crack/canker is defined as a dead section on the bark which first develops as an elongated water-soaked area with amber-colored gum. Infected tissue turns dark brown, and eventually the affected area becomes sunken. External lesions presented as staining or sap streaks on the stem surface where the *Ceratocytis* has invaded freshly damaged tissue. Both

cankers and external lesions can be associated with gummosis (the sticky, reddish gum that is produced by the tree and is not easily removed by rain) and exudate (the frothy product of fermentation that washes off in the rain). A tree was categorised as dead if all its phyllodes had turned brown or fallen from the tree and the stem was dry.

Tree survival time was defined as the time (weeks) from the first appearance of symptoms or signs of the disease to tree death.

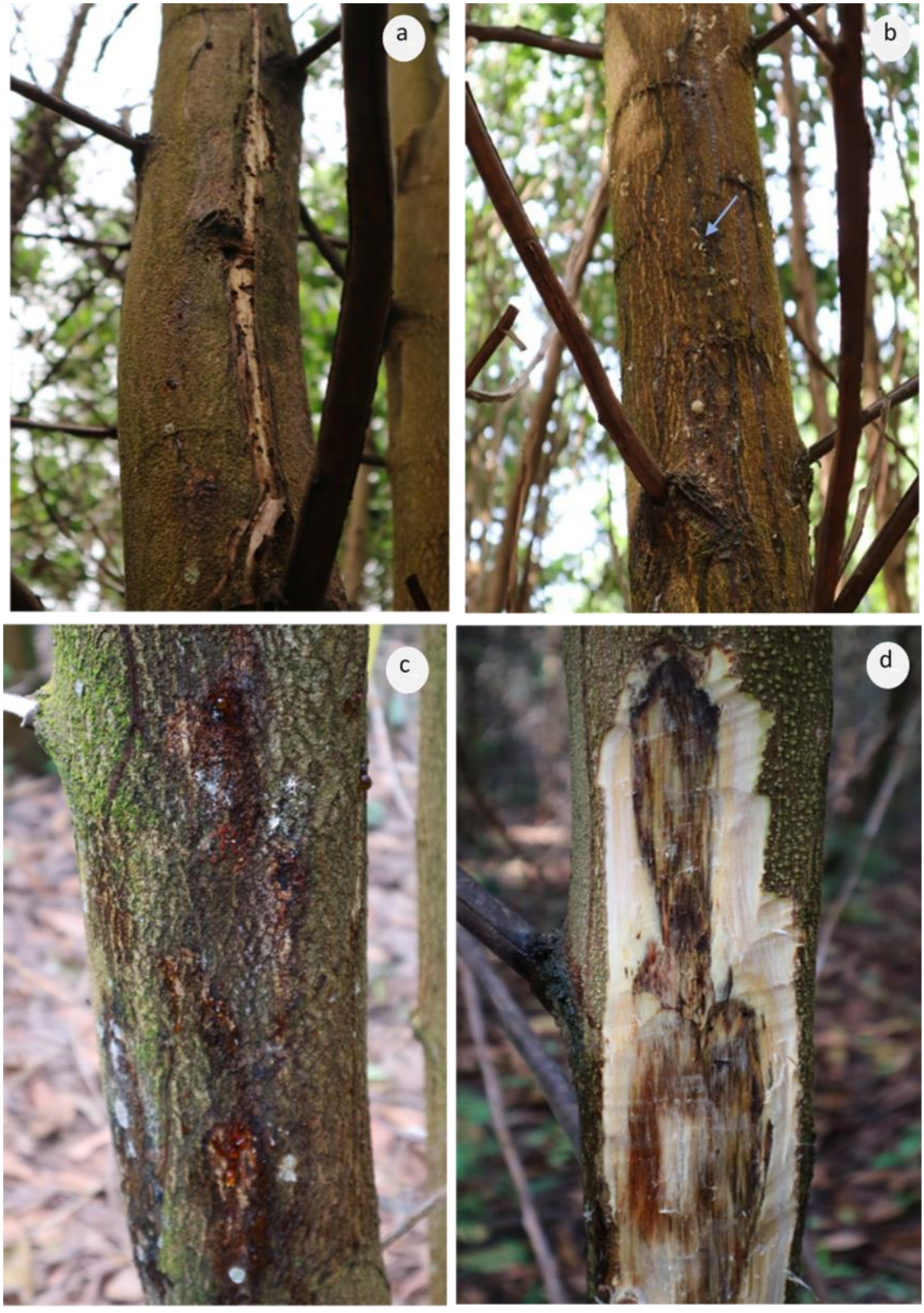

Figure VI-1 Ceratocystis wilt and canker disease symptoms and signs; a) bark stripping by monkeys, b) borer holes and frass, c) dark brown-black discoloration of bark (external lesion) and d) internal lesion (bark removed).

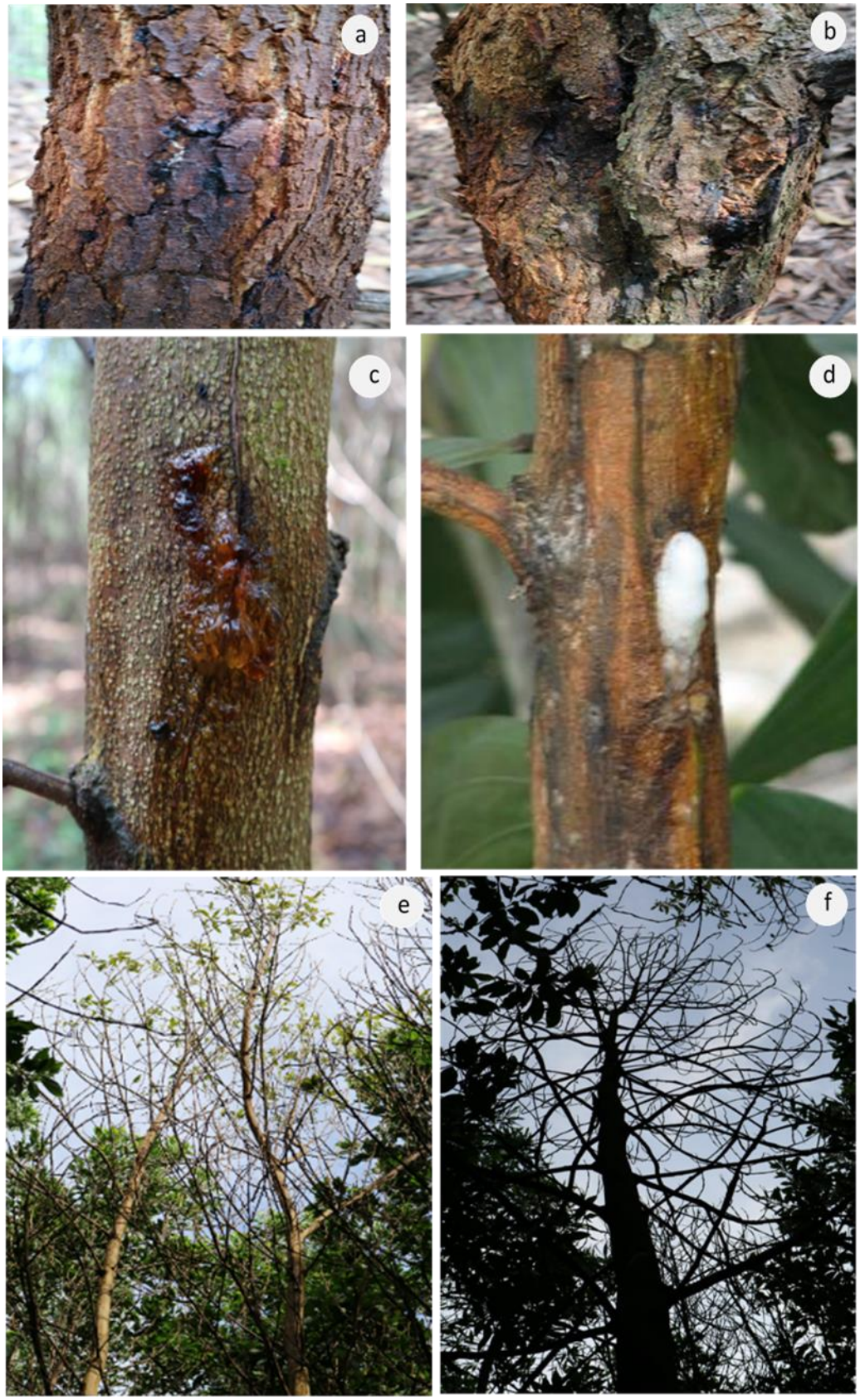

Figure VI-2 Ceratocystis wilt and canker disease symptoms and signs; a) crack b) canker, c) gummosis, d) frothy exudate, e) yellow and wilting leaves, and i) dead tree – stem is dry and all phyllodes have been lost.

VI.2.3. Leaf Area Index (LAI) measurement

Leaf Area Index (LAI) was defined by Watson (1947) as the total one-sided area of leaf tissue per ground surface area. A true measure of leaf area index is derived by harvesting leaves from the tree and determining dry mass in laboratory. The total dry mass of leaves then is converted into LAI (cm^2/g) by multiplying with the specific leaf area (SLA) (Bréda 2003). The effect of ceratocystis disease on the density of leaves in the canopy was estimated using a proxy measure for leaf area index applied each time the trees were assessed for disease or damage. This proxy measure was obtained using a visual guide to leaf area index (LAI) for eucalypt plantations (Cherry et al. 2002) modified for the acacia plantation. The proxy LAI was scored within values of 2 – 8 corresponding to the highest to lowest proportion of visible sky within the planted area. The LAI scores and corresponding canopy cover (in parentheses) were: 2.0 (<10%); 2.5 (10-20%); 3.0 (20-30%); 3.5 (30-40%); 4.0 (40-50%); 4.5 (50-60%); 5.0 (60-70%); 6.0 (70-80%); 7.0 (80-90%); and 8.0 (> 90%). The LAI value for each tree was measured using a mirror which was held at breast height (approximately 1.3 m) and by standing between two trees within a row and position the mirror to view the canopies of an additional two trees in both adjacent rows. In this position the mirror can reflect six trees overhead. The LAI was derived by looking down and positioning the mirror so that the assessor could see the target area of canopy overhead and estimate the proportion of the sky obscured by the tree canopy.

A frequency distribution of LAI scores for each assessment time and tree was plotted so that the distribution for healthy trees (no symptoms or signs) could be compared visually against the distributions for trees expressing ceratocystis disease. The trees expressing ceratocystis disease were then used to generate a frequency distribution of the LAIs observed for each category of disease symptom or sign of damage. The purpose of this plot was to observe the relative effect of a specific symptom of ceratocystis disease on LAI.

VI.3. Results

VI.3.1. Disease incidence and symptom progression

At the first assessment date, ceratocystis disease incidence, including the central, infected tree and a small proportion of the neighbouring trees that were symptomatic, was 22.2% for data combined from areas I-028 and J-060. Disease incidence across these areas increased from 22 to 41% between weeks 1 and 13 and plateaued between weeks 13 and 19 (Figure VI-3). All 15 of the trees selected for the centre of each plot had pin borer holes with frass, stem discoloration and cracks or canker.

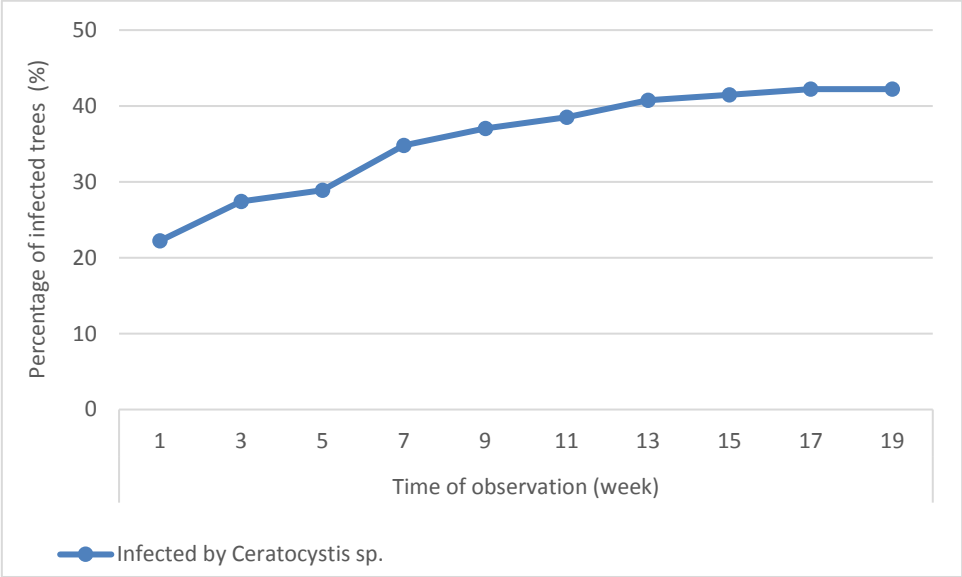

Figure VI-3. Temporal progression of ceratocystis disease incidence (DI) for *Acacia mangium* across planting areas I-028 and J-060.

Relatively high incidences of pin borer attacks were recorded in week 1 compared with other signs and symptoms. Over the ensuing weeks, new symptoms were observed in six to 14 trees on each visit, with new pin borer attacks recorded on all but two assessments (Figure VI-4).

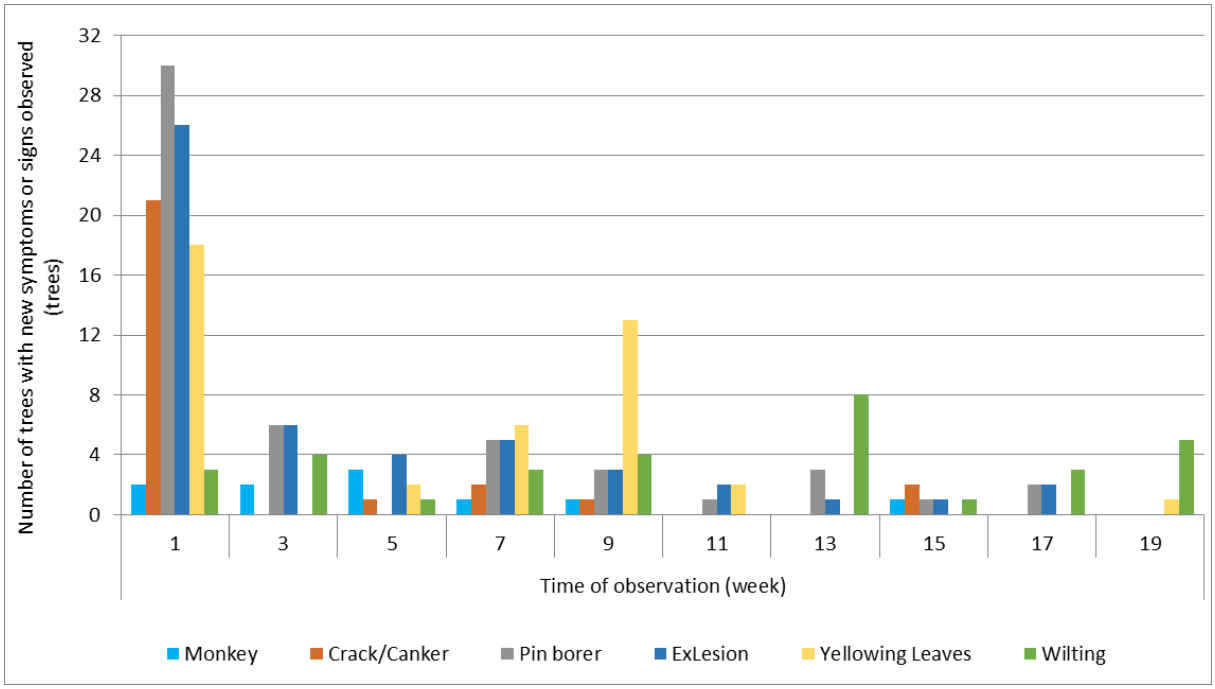

Figure VI-4. Timing of the first observation of ceratocystis disease symptoms in 135 *A. mangium* trees assessed fortnightly over 19 weeks. The figures for week 1 include the 30 trees that presented symptoms or signs in the initial survey. At subsequent dates, only new symptoms are recorded.

Pin-borer holes and bark discolouration (external lesion) were most frequently observed, at a higher incidence than other categories of observation (Figure VI-5). Pin-borer holes were observed before any other sign of disease in 29 trees and were the most frequently noted first sign. Fifty-one trees (approximately 38% of all trees assessed) were showing pin-borer holes and bark discoloration at the end of the assessment period. The incidence of monkey damage was relatively low throughout the survey and was steady at 7.4% from weeks 15 to 19 (Figure VI-5).

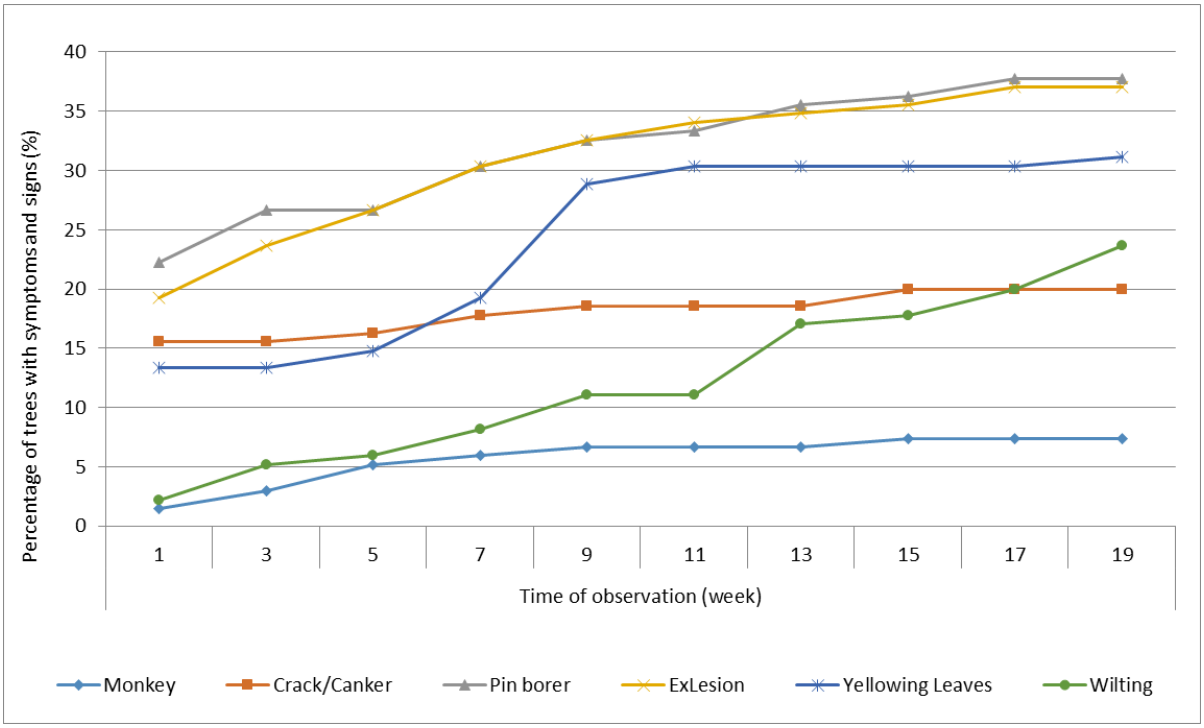

Figure VI-5. Temporal progression of the percentage of *Acacia mangium* trees (n=57) with the six different categories of symptoms or signs.

Most trees with only pin borer holes, external lesion and crack/canker symptoms were observed to survive for at least 18 weeks (Figure VI-6). Trees with yellowing phyllodes mostly survived for at least 10 weeks. Trees with wilting phyllodes had a relatively short survival time. The majority of trees died within 2-4 weeks of this symptom first appearing (Figure VI-6).

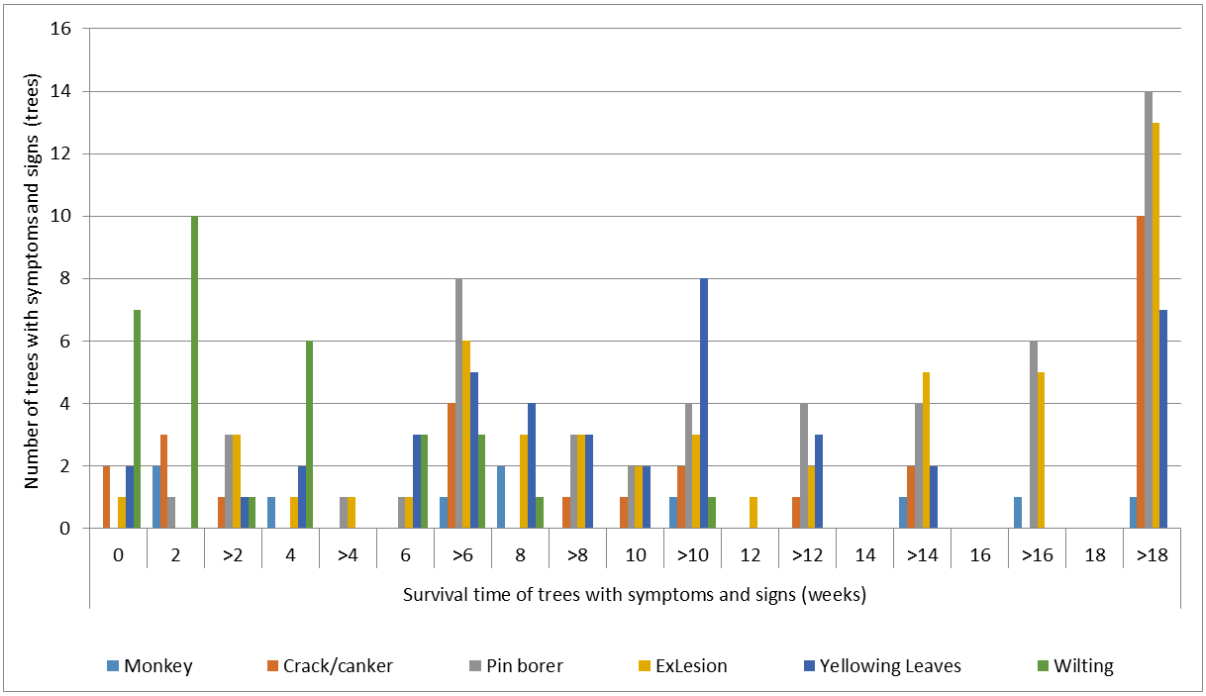

Figure VI-6. Survival time of the 57 infected *Acacia mangium* for the 6 categories of symptoms and signs monitored for ceratocystis wilt and canker disease. Survival time was the length of time from the first appearance of that symptom or sign until tree death. Where symptoms were present in the initial survey or where the tree was still alive at the final survey, the exact survival period could not be determined so is included as >n, n being the minimum number of weeks that the tree survived.

VI.3.2. Disease impact on leaf area index (LAI)

There was a tendency of trees expressing ceratocystis disease to have lower LAI scores of 4.5-5.5 (Figure VI-7) associated with a slow decline in LAI (Figure VI-8) but the pattern was not always consistent. LAI in healthy trees was higher (Figure VI-8) without a marked gradual decline. Around 5.2% of 57 infected trees had LAI scores of 2.5 or lower by the time the phyllodes were wilting (Figure VI-7).

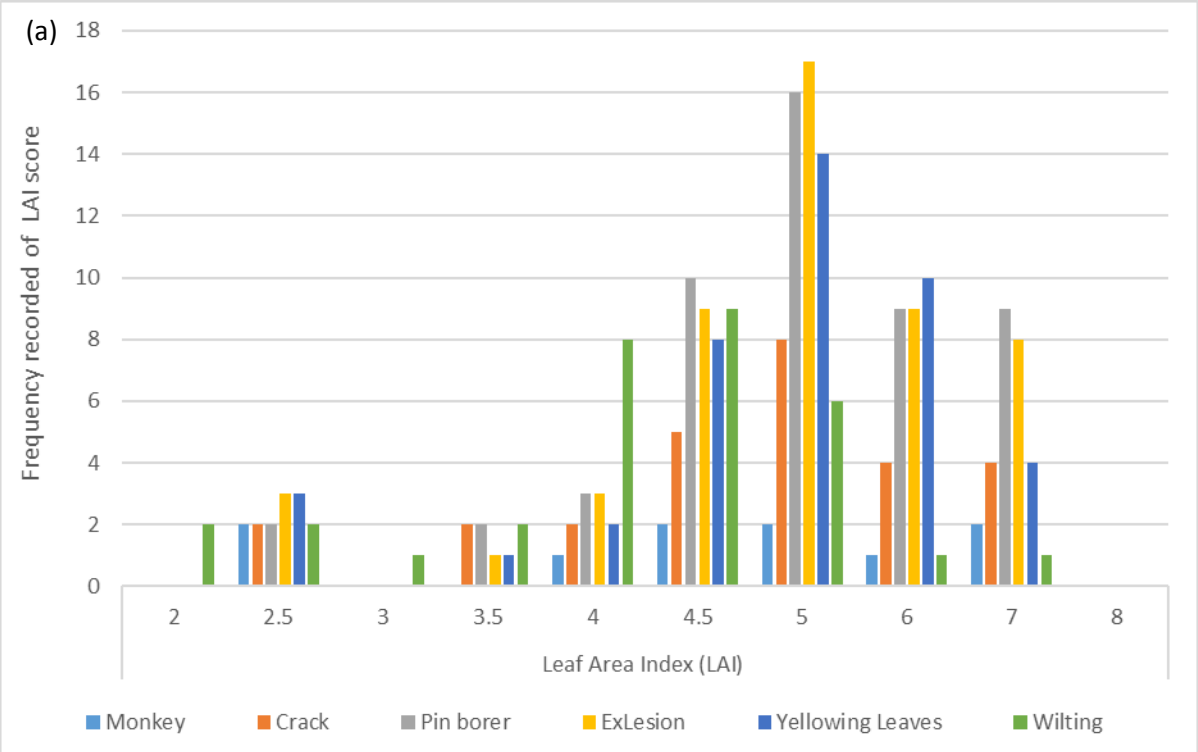

Figure VI-7 LAI scores recorded on *Acacia mangium*; frequency distribution of LAI in infected trees at the first appearance of the given symptom; the LAI was typically 5 at the first appearance of external lesion, pin borers, or phyllode yellowing and 4.5 at the first sign of wilting.

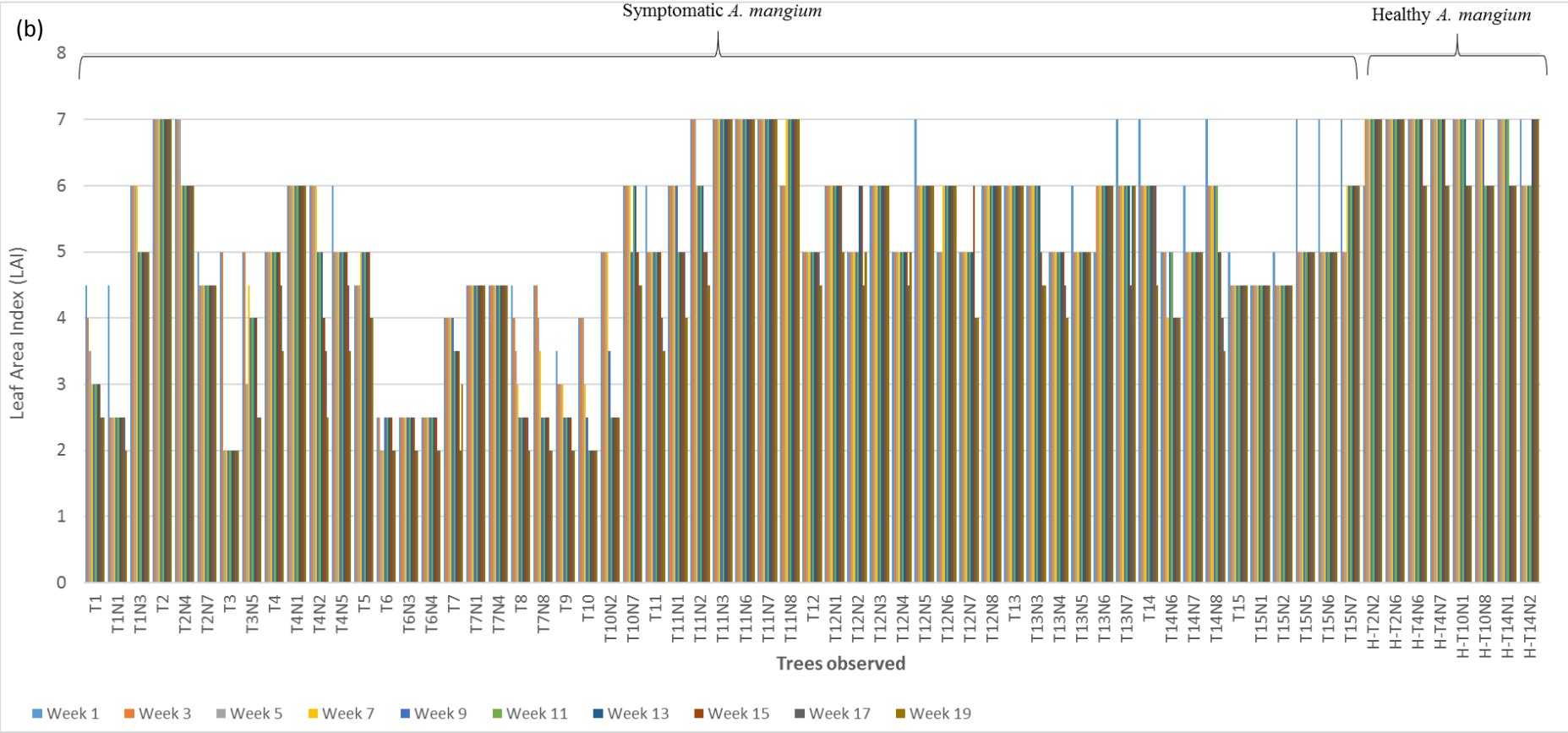

Figure VI-8 LAI for all infected centre trees and new symptomatic trees (57 trees) over the observation period, compared with corresponding LAI values in eight representative healthy trees.

VI.4. Discussion

Wounding is a key factor promoting successful infection of trees by *Ceratocystis* spp. (Kile 1993). In this study, holes created by pinhole borers were observed before any specific signs or symptoms of ceratocystis disease. These insects were observed wounding the bark and preparing tunnels through the sapwood and sometimes heartwood with subsequent gallery development by the larvae. Several species of pinhole borers in the genera *Xyleborus*, *Xyleborinus*, *Euwallacea*, *Hyphothenemus*, and *Xylosandrus* have been found in association with acacias in Sumatra plantation (Wagner Souza, unpublished). However, species of *Xylosandrus* and *Xyleborus* are the predominant pinhole borers in *Acacia* plantations in Indonesia (Nair 2000). These pinhole borers are members of the ambrosia beetle or fungal-feeding guild (Kirkendall & Faccoli 2010; Beaver et al. 2014) which bore into the trees, creating galleries through the wood for breeding and cultivation of fungi on gallery walls or in specialized beds to provide food for the larvae (Klepzig & Six 2004; Beaver et al. 2014). However, there is an interaction among ambrosia beetle, associated fungi and the hosts (trees) which can enhance fungal growth for feeding the larvae (Freeman et al. 2016). Most ambrosia beetles attack trees which are already stressed, dying or dead. Tree stress may have been initiated by drought, flooding, wind damage, extreme temperature or very poor silvicultural practices (Carrillo et al. 2014). However, some ambrosia beetles, such as *Xyleborus glabratus,* attack healthy trees (Kühnholz et al. 2001). As ambrosia beetles represent a guild with a common feeding strategy which has evolved multiple times, the group is not monophyletic (Hulcr et al. 2017), and so general statements may not be accurate when applied to specific beetle/host species interactions. A strong association of ambrosia beetles with *Ceratocystis*-infected *Acacia* trees has also been noted in Malaysia (Brawner et al. 2015). In Brazil, an association between *Ceratocystis fimbriata, Xyleborus affinis* and mango wilt has been observed, with the pathogen isolated from beetles and their frass (Souza et al. 2013). Tree stress

due to silvicultural practices in *Acacia* plantation such as pruning, or singling may attract ambrosia beetle to attack the trees (Tarigan et al. 2011b). However, more detailed studies and observations are needed to confirm this association.

Ceratocystis spores (ascospores and conidia) may be transported to wounds by insects, animals including humans or air currents (Harrington 2013). Insects, including pinhole borers, are known to play a role as vectors in other *Ceratocystis* diseases (Harrington 2013). *Ceratocytis* spp. produce masses of sticky sexual spores (ascospores) on the stem surface which are resistant to removal by wind or water (Harrington 2013). However, these spores easily adhere to fungal feeding insects or to insects attracted by the fruity odours and fermentation exudate produced by *Ceratocystis* and associated bacterial growth in acacia trees (Brawner et al. 2015). Insect borers are known to play a role in the spread of other *Ceratocystis* diseases (Harrington 2013). The sticky ascospores become attached to the insect bodies and are transported to other trees that subsequently become infected and develop disease symptoms. Alternatively, insect frass, which may contain viable chlamydospores or aleurioconidia, is extruded from borer holes as galleries are excavated, and may be blown by wind to neighbouring trees (Friday et al. 2016).

However, monkeys are also implicated in disease spread due to the creation of wounds on trees and the potential for simultaneous transmission of *Ceratocytis* spores. Anecdotally, a high incidence of ceratocystis disease in *A. mangium* has been associated with high levels of bark stripping by monkeys. The relatively low level of monkey attack observed in this study might be explained by the high proportion of *Eucalyptus* trees in the areas surrounding the study site. *Eucalyptus* is considered a non-target tree for monkeys and are relatively free from monkey attack. Indeed, *Acacia* and *Eucalyptus* plantations in the region are being maintained in a 20:80 proportion (Wong Ching Yong, pers. comm.) to deter monkey attack in *Acacia*.

However, this study shows that the disease incidence increases rapidly over a short time in the absence of monkey damage and its onset appears associated with wounds created by borers.

Signs and symptoms of Ceratocytis infection in *A. mangium* started with wounds on stems or branches which, in our study, were created by pinhole borers and, to a lesser extent, monkeys. External lesions (stem discolorations) were observed subsequently. Cracks, sunken cankers, gummosis and fermented exudate also followed the borer holes and monkey damage. These symptoms culminated in yellowing of the acacia phyllodes, wilting and death. These symptoms are similar to reports in other woody trees infected by *Ceratocystis,* including mango (Araujo et al. 2014); *Prosopis cineraria* and *Dalbergia sissoo* (Al Adawi et al. 2013); *Metrosideros polymorpha* (Barnes et al. 2018); *Eucalyptus* sp. (Alam et al. 2017); and *Platanus acerola* (Pilotti et al. 2016). Though detailed timelines of symptom progression are not provided, yellowing and wilting or dropping of leaves are frequently noted as the final stages of the disease (Al Adawi et al. 2006; Asner et al. 2018). The average time from inoculation until death in 5 year old *P. acerola* inoculated with *C. platani* ranged from 123 to 396 days and depended on the season of inoculation (Pilotti et al. 2016), whereas 12-month-old *A. mearnsii* had a mortality rate of 4-44% within 6 weeks of artificial inoculation with *C. albifundus* (Roux et al. 1999). Seasonal variation is not expected to have a strong role in the *Ceratocystis: Acacia* interactions in Indonesia as the temperature does not vary as significantly as in temperate climates and the trees do not go dormant. In mango, death of 2 year old trees usually occurred within 6 months of the first symptom appearance, with gummosis the first symptom reported following artificial inoculation (Al Adawi et al. 2006).

Acacia mangium trees infected by *C. manginecans* with severe symptoms such as yellowing leaves and wilting typically died within 2-4 weeks of developing these symptoms. In contrast, trees with 'early' symptoms only (external lesion, cracks/cankers or gummosis) often survived for over 18 weeks. However, survival time following *Ceratocystis* infection may

also be influenced by tolerance or resistance of trees against ceratocystis disease or by plant age (age-related resistance (ARR). Plant tolerance or resistance against pathogens increases in the older plants due to an increase in production of defence-associated compounds such as salicylic acid (SA), jasmonic acid (JA) or ethylene (ET) (Panter & Jones 2002; Eyles et al. 2010). Disease resistance or tolerance is considered as the most promising strategy for managing ceratocystis disease (Kile 1993; Harrington 2013; Brawner et al. 2015).

Ceratocystis disease in acacia trees was associated in this study with reductions in leaf area index (LAI) as the disease progressed but prior to the final stages of yellowing, wilting and death. The LAI did not decline in a small subset of the infected trees, this could be investigated as a potential indicator of genetic tolerance. LAI can be a useful factor in the detection of tree stress caused by biotic factors such as pests and diseases (Zheng & Moskal 2009; Mora et al. 2016). Ground surveys of plantation health are time-consuming and costly. Aerial detection based on an analysis of canopy cover have been used to monitor and map ceratocystis wilt disease in plantation eucalypts in Brazil (Souza et al. 2015) using images acquired by cameras mounted on Unmanned Aerial Vehicles (UAVs). Analysis of UAV imagery reliably and accurately distinguished between healthy and infected trees (Souza et al. 2015).

In summary, the rapid development of ceratocystis wilt and canker disease in this study appeared to be associated with the activity of pin borers, most probably acting as vectors. An approximate time line was established for the progression of the disease including a reduction in leaf area index (LAI) relative to healthy trees. This now provides an opportunity to explore the use of aerial monitoring of ceratocystis wilt and canker disease in *A. mangium.*

VII. General Discussion

Ceratocystis wilt and canker disease has emerged as a severely damaging disease in acacia plantations in SE Asian countries and has seriously impacted the sustainability of acacia wood production (Nambiar & Harwood 2014). In SE Asia, *Acacia* species are mainly used as raw material for pulp and paper industries (Griffin et al. 2011). Efforts to combat this disease have focused on the selection of tolerant trees and the avoidance of wounding during silvicultural practices (Marthin Tarigan, pers. comm.). The studies in this thesis were targeted at supporting the screening efforts and exploring a new option for *Ceratocystis* management i.e. biological control.

The studies therefore focussed on; 1) developing and validating a potential rapid tolerance/resistance screening protocol; 2) exploring the diversity of endophytic bacteria in *Acacia mangium*; 3) testing the potential of these bacteria as biological control agents against *Ceratocystis manginecans,* and 4) establishing a timeline for the appearance of different symptoms and signs of *C. manginecans* in *A. mangium* in order to better understand disease epidemiology and to be able to effectively monitor disease management field trials.

VII.1. Main findings on the study

VII.1.1. A rapid protocol for resistance screening of *Acacia* species against ceratocystis wilt and canker disease

The inoculation of very young trees in pots is a traditional method for screening trees to identify resistant to germplasm (Roux et al. 2000b; Roux et al. 2004b; Van Wyk et al. 2010). Compared with field inoculation tests, the inoculation of potted plants is potentially less costly in time and resources, avoids introducing more disease inoculum into the field and, depending

on quarantine requirements, is a method to evaluate the pathogenicity of non-native isolates (Van Wyk et al. 2010). There is an argument that greenhouse experiments with young trees may express different results to field experiments (Van der Westhuizen et al. 1992). Field trials are an important component of breeding programs, but a rapid, preliminary screening can expedite selection of more tolerant material. There can also be greater replication and scope of screening with pot trials and the inoculations can be carried out under more stringently controlled conditions. Inoculated pot trials have been adopted for many of the recent screening trials carried out with *Ceratocystis* and *Acacia* because of the urgent need to identify tolerant germplasm in an apparently super-susceptible species (*A. mangium*).

The potted plant inoculation procedure with *Acacia* and *Ceratocystis* took approximately 18 weeks starting from seedling preparation to the end of the trial. In this study it took approximately 5 weeks from inoculation of potted plants until the trial could be harvested and internal lesions measured. The low levels of exploitable resistance, especially in *A. mangium* (Brawner et al. 2015), mean that a very large number of genotypes will have to be screened to maintain an effective resistance breeding program. Consequently, the 4-5 months required for a pot trial will be too slow in many instances, so a rapid screening method for testing material coming from field genetic trials that avoids inoculation in the field will be valuable.

My study compared the inoculation of potted plants with two potential rapid screening procedures; inoculation of acacia phyllodes and stem segments. It was difficult to avoid desiccation and contamination in the stem segment inoculation experiments. This desiccation shortened the time available to assess the lesions on the stem segments. The drying out of stem segments may therefore skew results, influencing spore germination or mycelial growth in the stem (Zalasky 1965; Keane & Kerr 1997) resulting in shorter lesions. Attempts to prevent the desiccation using wax on stem segment ends, standing the segments in moistened cotton or

sand showed that wax was most effective in reducing drying but was only effective for a maximum of two weeks. While the results with stem segments were not as discriminatory as those with inoculated potted plants and phyllodes, they could potentially be improved by using larger, woodier stem segments. Larger stem segments may mean slower desiccation, providing adequate time to assay the lesions on the stem segments, and have been used successfully for testing disease resistance in trees (Van Wyk et al. 2010). However larger segments will be far less easy to harvest in the field and to manipulate in the laboratory.

The phyllode inoculation assay takes far less time and effort to establish than the pot trials or stem segments and needs only 2-3 weeks to complete. Results with this procedure showed a correlation with potted plant inoculations with an almost identical trend of disease incidence (lesion and necrosis) on inoculated stems and phyllodes. Other authors have also shown meaningful correlations between lesion development on inoculated tree stems and that on inoculated leaves for *Ceratocystis* host-pathosystems (Newhouse et al. 2014; Magalhães et al. 2016). Preliminary experiments also indicated that there is no difference in results between phyllodes inoculated immediately after collection and those inoculated the following day after overnight storage at 4 °C. This is important given the distances that may be required to collect phyllode material.

The *Ceratocystis* isolates which were used in this study varied in their aggressiveness. Knowledge of pathogen variability is crucial to interpreting inoculation assays for host resistance. Previous studies of *Ceratocystis* aggressiveness have indicated a wide variation in this characteristic and some isolates of acacia *Ceratocystis* may also be avirulent (Baker et al. 2003; Harrington et al. 2011). While there were differences in the aggressiveness of the isolates this did not appear to influence the nature of the correlations between the potted plant and the phyllode inoculations. Both the mycelium plug size and spore suspension concentration in this study were adequate for producing stem lesions. The inoculum form only produced a difference

in aggressiveness in the early stages of infection, with higher disease measures from mycelial inoculum, probably due to an immediate start of the infection process by mycelium while spores in suspension need to germinate before starting to infect the plant tissues.

My study established that the phyllode testing method has potential as a rapid screening protocol and that it works with isolates of different aggressivity and with a certain concentration of spores. However, my study evaluated the phyllode screening method with two *Acacia* species (*A. crassicarpa* and *A.* hybrid) evidently more tolerant to *Ceratocystis* infection compared to *A. mangium.* A large validation assay is required to see how nuanced and repeatable the phyllode test is – does it only reflect marked differences in tolerance? Acacia germplasm found to be tolerant to *Ceratocystis* in potted plant inoculations has been planted out in the field. It will be informative to compare results from the natural inoculation of these trees, previous data on their level of tolerance in potted plant inoculations and from phyllode tests, either historical data or new data created with phyllodes collected and inoculated from the current field trials.

VII.1.2. Diversity of culturable endophytic bacteria from *Acacia mangium* Wild

In total 276 isolates of endophytic bacteria (EB) were isolated from roots, stems and phyllodes of *Acacia mangium.* DNA sequence analysis grouped them into five clusters corresponding to the phyla Actinobacteria and Firmicutes, and the three classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria in the phylum Proteobacteria. Among these five clusters, Firmicutes were isolated most frequently from acacia trees. This phylum includes the genus *Bacillus* which had the widest distribution in roots, stems and phyllodes of *A. mangium*. These results are similar to previous observations of EB diversity in

woody trees such as European deciduous and coniferous trees (Izumi et al. 2008), moso bamboo (*Phyllostachys edulis*) (Han et al. 2009) and *Eucalyptus* (Ferreira et al. 2008a).

Higher numbers of EB were obtained from young *A. mangium*, particularly trees less than one year old, and decreased gradually in the older trees (2 to 5 years old). This result indicated that the numbers of EB in plant tissues can be influenced by plant age as well as plant organ or tissue. Similarly, a study conducted by Vendan et al. (2010) in ginseng, demonstrated higher incidence and diversity of EB in plants under four years old, and reduced drastically in the mature plants. Plant roots attract the EB community by providing exudates or chemical compounds such as soluble sugars, proteins, amino acids, organic acids and other nutrients (Compant et al. 2005; Renaut et al. 2005). The concentration and type of exudates vary with the maturation stage of plants which then may impact the population and variability of EB in the plant tissues (Ferreira et al. 2008b).

The number of EB isolated from acacia roots was double that of bacteria derived from stems and phyllodes. Their diversity on roots is also more complex; all of the 25 genera which were isolated from *A. mangium* were obtained from acacia roots. Among these genera, *Bacillus* and *Burkholderia* were the most frequently isolated. The higher incidence and diversity of EB in root tissues compared to stems and phyllodes may be because roots are the main entry point for EB, through epidermal conjunction or wounds which may be created by plant growth or through root hairs (Sprent & De Faria 1988). Plant roots also produce copious exudates that attract and provide nutrients for EB.

VII.1.3. Endophytic bacteria from *Acacia mangium* as potential biological control agents against ceratocystis wilt and canker disease

More than 50% of endophytic bacterial isolates from *A. mangium* (157 from 278 isolates) significantly inhibited growth of *Ceratocystis manginecans in vitro.* While most of these isolates competed for space and nutrients, six EB isolates produced antimicrobial metabolites as shown by tests based on culture supernatant. The antimicrobial activity was recognised through the clear zone with no *C. manginecans* growth surrounding the EB supernatant. These antimicrobial metabolites supressed *C. manginecans* growth through an antibiosis mechanism which has been considered as the main mode of inhibition by EB due to their greater likelihood of producing various enzymes and antibiotics (Shali et al. 2010; Brader et al. 2014).

The endophytic bacteria which expressed metabolite activity against *Ceratocystis* were identified as belonging to six genera; *Paenibacillus*, *Staphylococcus*, *Lysinibacillus*, *Pantoea*, *Ralstonia* and *Ochrobactrum*. Among these genera, *Paenibacillus* and *Lysinibacillus* demonstrated the highest metabolite activity, enabling them to block *Ceratocystis* growth. These genera belong to Firmicutes, members of which are known to produce chitinolytic enzymes (Williamson et al. 2000; Brzezinska et al. 2014), and can secrete several antimicrobial enzymes including glucanases, lipases, chitinases, cellulases and proteases which can be useful to destroy eukaryote cell walls (Grady et al. 2016; Naureen et al. 2017).

Evaluation of five techniques for inoculating EB into *A. mangium* (including; soaking seeds in bacterial suspension, pouring the bacterial suspension onto germinating seedlings, spraying germinating seedlings with the suspension, dipping stem cuttings into and spraying stem cuttings with bacterial suspension) revealed that either pouring or spraying germinating acacia seedlings/cuttings were more successful than soaking seeds or dipping cuttings in the bacterial suspension. This result indicates that EB are more likely to enter the acacia tissues

through the root system during the process of germination and subsequent root growth. The wounds that naturally occur in the germinating radicles or epidermal conjunctions, root hairs and sites of root branches can be used as bacterial entry points as well as providing a food source for bacteria through leakage of plant exudates (Hallmann et al. 1997).

In this study, spraying EB suspension onto acacia stem cuttings before rooting was not a successful delivery method. This may have been because the bacteria were unable to colonise the plant surface due to the lack of roots and exudate as a food source for EB to survive before entering the plant tissues (Hallmann et al. 1997). The deployment of EBs is considered likely to be a part of an integrated management strategy that will rely heavily on the development of resistant and tolerant *Acacia* clones. Clonal material will, of necessity, be propagated by stem cuttings. In this case, delivery of EB into plant stem cuttings is likely to be more successful through pouring or spraying once the root has grown.

VII.1.4. The temporal development of ceratocystis disease signs and symptoms on *Acacia mangium*

The development of *C. manginecans* signs and symptoms were monitored over a 19-week period. The symptoms and signs of *C. manginecans* in *Acacia* spp. have been described and are very similar to ceratocystis diseases in other trees but, before my study, information about their temporal appearance and survival time of infected trees was anecdotal. The incidence of disease in areas monitored doubled over the 19-week period. While it is well known that *C. manginecans* causes serious damage to acacia trees in Vietnam (Thu et al. 2012), Malaysia (Brawner et al. 2015) and Indonesia (Harwood & Nambiar 2014a) and that this happens rapidly, knowing that incidence can double in under 5 months demonstrates the potential sudden impact of this disease.

Bark stripping by monkeys and stem damage by borers were associated, in this study, with the initial stages of disease development. Pinhole borers were observed wounding the bark and preparing tunnels through the sapwood and sometimes heartwood which were expanded into galleries by developing larvae. The preference of pinhole borers for *A. mangium* over *A. crassicarpa* has been suggested as a possible reason for the greater impact of *C. manginecans* on *A. mangium* than on *A. crassicarpa* (Tarigan et al. 2011b). The observations of borers were typically followed by bark lesions, bark cracking, cankers, gummosis and a frothy exudate with a fruity odour on the stem. These symptoms persisted, in some cases for months, before the leaves turned yellow, wilted or fell and the tree ultimately died.

Recent developments in remote sensing technologies and big data modelling techniques can now provide spatially explicit, quantitative solutions for management or research objectives that are more accurate than manual field-based assessments of tree damage or airborne visual mapping (Stone & Mohammed 2017). Knowledge of the phenology of the damaging agent and associated host response such as provided in this study has implications for the optimal timing of data acquisition as well as the optimal reference data required for classifying or training remotely sensed data.

VII.2. Conclusions and future research

1. The phyllode screening protocol can be considered as a rapid screening technique for genetic resistance in *Acacia*. The most effective strategy to control ceratocystis wilt and canker disease in forest plantations is through planting resistant or tolerant trees. Thus, a rapid screening protocol is an important tool to accelerate the screening of a wide variety of germplasm. The technique however requires further validation.

2. Diverse endophytic bacteria (EB) which produce antimicrobial metabolites can be obtained from *A. mangium*. Considering their potential as biological control agents (BCAs) of plant diseases through direct action (competition and antibiosis) or induced systemic resistance (ISR), these beneficial bacteria can be used as a complementary strategy to assist in control of ceratocystis disease. Introducing EB with the ability to trigger plant defences against pathogens such as *C. manginecans* may be more effective in controlling this disease as they can stimulate the plant's innate defences against this disease. Further work in this area is required to determine the effectiveness of biological control *in planta* and, if effective, the duration of the control.
3. Resistant and tolerant trees are more likely to be obtained through screening and breeding programs which then produce clonal materials or hybrids. These hybrids are usually propagated through stem cuttings. Based on this study result, the most effective techniques to introduce them into *A. mangium* were through pouring and spraying EB suspension onto germinating seedlings. Considering this result, for plant materials which are propagated through stem cuttings, EB delivery is better deferred until the stem cuttings have grown roots, though further trials will be necessary to determine the optimum stage at which this step should be carried out.
4. Wounding of trees by pin-borer insects was observed to play an important role in *Ceratocystis* epidemiology. Further research and observation in terms of insect taxonomy, their associations with acacia trees (hosts) and *Ceratocystis*, as well as other fungal associations need to be explored.
5. Small unmanned aerial vehicles (UAVs) can now carry miniaturised hyperspectral sensors in addition to the simpler multispectral cameras. The preliminary observations in my study can inform the timing of imagery acquisition and data analysis to spectrally characterise the progression of the disease. It is also necessary to explore other potential

Ceratocystis management strategies such as breeding for resistance and introducing beneficial microorganisms as biological control agents (BCA). The ability to monitor genetic trials or the success of the other disease management strategies will be crucial to reducing the impact of *C. manginecans*.

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Appendixes

Appendix 1: Isolated endophytic bacteria from *A. mangium* and inhibiting ability (%) against *C. manginecans* through contact and non-contact protocol in the dual culture (in-vitro) assay. (NA; Nutrient Agar medium and LB; Luria Bertani medium).

EB Code	Isolating Date	Plant source					Isolation area	Isolating medium	Inhibiting ability against *C. manginecans* (%)	
		Species	Age	Root	Phyllode	Stem			Contact	Non-contact
EB-5	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	LB Medium	82.86	72.61
EB-6	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	LB Medium	77.24	70.18
EB-33	26/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	77.41	70.07
EB-254	29/05/2015	*Acacia mangium*	6 Months	v			Pelalawan KCN-2	LB Medium	49.31	63.08
EB-93	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	86.36	63.03
EB-256	29/05/2015	*Acacia mangium*	6 Months	v			Pelalawan KCN-2	LB Medium	53.10	60.24
EB-161	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	53.36	58.90
EB-255	29/05/2015	*Acacia mangium*	6 Months	v			Pelalawan KCN-2	LB Medium	63.79	58.33
EB-56	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	3.86	57.63
EB-1	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	NA Medium	53.71	57.39
EB-241	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	NA Medium	85.53	56.74
EB-31	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	69.04	56.04
EB-223	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	LB Medium	84.31	55.94
EB-253	29/05/2015	*Acacia mangium*	6 Months	v			Pelalawan KCN-2	NA Medium	74.48	55.61
EB-238	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	87.23	55.28
EB-166	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	LB Medium	61.76	55.13
EB-263	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	68.97	54.96
EB-17	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	86.90	54.10
EB-201	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.04	53.42
EB-64	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	77.07	52.78
EB-273	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	NA Medium	84.65	52.41
EB-158	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	75.21	52.34
EB-73	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	83.13	51.72
EB-152	25/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	NA Medium	34.87	51.35
EB-244	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	LB Medium	86.38	50.79
EB-153	25/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	NA Medium	54.60	50.68
EB-7	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	68.86	50.00
EB-34	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	13.81	50.00
EB-226	29/05/2015	*Acacia mangium*	2 years			v	Baserah compartement I027	LB Medium	86.00	49.80
EB-274	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	NA Medium	82.67	49.43
EB-202	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.04	49.36
EB-171	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	30.25	49.25
EB-196	2/05/2015	*Acacia mangium*	4 years		v		Baserah compartement J015	NA Medium	82.71	49.12
EB-53	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	53.65	48.44
EB-145	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	55.65	48.03
EB-12	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	74.29	47.87
EB-212	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	84.71	47.34
EB-170	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	26.05	45.98
EB-108	24/3/2015	*Acacia mangium*	10 Months			v	Mandau Compartement C009	NA Medium	65.66	45.95
EB-235	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	85.11	45.54
EB-250	29/05/2015	*Acacia mangium*	4 Years			v	Baserah compartement I048	NA Medium	82.41	45.25
EB-116	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	46.86	44.85
EB-146	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	63.31	44.44
EB-159	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	52.52	44.39
EB-184	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	31.79	44.26
EB-138	28/3/2015	*Acacia mangium*	1 year	v			Kerinci Research Nursery-KRN	NA Medium	72.18	44.17
EB-245	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	LB Medium	71.03	44.12
EB-111	24/3/2015	*Acacia mangium*	10 Months			v	Mandau Compartement C009	NA Medium	50.72	44.06
EB-156	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	86.55	43.59
EB-68	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	81.89	43.28
EB-213	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	31.76	42.94
EB-242	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	NA Medium	86.81	42.79
EB-186	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	LB Medium	25.13	42.77
EB-149	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	52.02	42.64
EB-214	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	73.33	42.38
EB-198	2/05/2015	*Acacia mangium*	4 years		v		Baserah compartement J015	NA Medium	80.37	41.96
EB-181	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	22.05	41.62
EB-66	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	69.55	41.52
EB-67	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	81.07	41.38
EB-70	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	81.95	41.38

Appendix 1 (continued)

EB Code	Isolating Date	Plant source					Isolation area	Isolating medium	Inhibiting ability against *C. manginecans* (%)	
		Species	Age	Root	Phyllode	Stem			Contact	Non-contact
EB-142	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	69.76	41.30
EB-137	28/3/2015	*Acacia mangium*	1 year	v			Kerinci Research Nursery-KRN	NA Medium	85.48	40.99
EB-144	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	67.34	40.85
EB-275	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	NA Medium	82.67	40.74
EB-27	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	21.83	40.48
EB-77	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	78.60	39.91
EB-150	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	52.82	39.44
EB-40	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	63.60	38.24
EB-187	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	LB Medium	58.46	37.82
EB-180	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	33.85	36.84
EB-164	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	20.17	36.65
EB-109	24/3/2015	*Acacia mangium*	10 Months			v	Mandau Compartement C009	NA Medium	54.11	36.60
EB-84	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	87.06	35.63
EB-188	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	LB Medium	50.26	35.27
EB-10	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	16.00	35.22
EB-239	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	82.98	34.88
EB-236	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	85.11	34.46
EB-197	2/05/2015	*Acacia mangium*	4 years		v		Baserah compartement J015	NA Medium	73.83	33.50
EB-41	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	55.23	33.07
EB-278	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	62.87	32.98
EB-225	29/05/2015	*Acacia mangium*	2 years			v	Baserah compartement I027	LB Medium	84.26	32.88
EB-220	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	88.24	32.62
EB-29	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	29.26	32.47
EB-9	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	20.67	32.40
EB-3	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	NA Medium	73.71	31.58
EB-139	28/3/2015	*Acacia mangium*	1 year	v			Kerinci Research Nursery-KRN	NA Medium	83.84	31.14
EB-183	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	38.97	31.10
EB-271	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	83.17	31.02
EB-258	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	NA Medium	74.14	30.77
EB-74	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	44.03	30.50
EB-148	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	45.97	30.48
EB-270	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	NA Medium	82.67	30.32
EB-163	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	74.79	29.91
EB-151	25/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	NA Medium	46.37	29.63
EB-237	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	83.83	29.56
EB-143	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	58.87	29.37
EB-172	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	27.18	28.66
EB-216	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	76.08	28.57
EB-18	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	73.36	28.32
EB-123	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	44.93	28.26
EB-48	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	70.39	28.16
EB-141	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	31.05	28.03
EB-16	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	68.56	27.59
EB-233	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	84.26	27.56
EB-157	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	72.69	27.45
EB-13	25/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	72.57	27.27
EB-277	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	85.15	27.21
EB-110	24/3/2015	*Acacia mangium*	10 Months			v	Mandau Compartement C009	NA Medium	47.80	27.07
EB-272	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	82.67	26.96
EB-42	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	25.52	26.83
EB-115	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	56.52	26.44
EB-176	30/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	LB Medium	72.82	25.94
EB-211	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	2.75	25.55
EB-104	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	20.00	25.33
EB-185	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	64.62	25.00
EB-168	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	LB Medium	81.93	23.40
EB-26	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	12.66	23.04
EB-45	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	68.20	22.64
EB-243	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	NA Medium	86.38	22.41
EB-60	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	82.70	21.82
EB-194	2/05/2015	*Acacia mangium*	4 years		v		Baserah compartement J015	NA Medium	81.78	21.59
EB-174	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	72.82	21.57
EB-140	28/3/2015	*Acacia mangium*	1 year	v			Kerinci Research Nursery-KRN	NA Medium	80.24	21.43
EB-120	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	55.56	21.05
EB-219	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	88.24	20.69
EB-51	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	75.97	20.35
EB-133	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	66.10	20.21
EB-134	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	46.77	19.89

Appendix 1 (continued)

EB Code	Isolating Date	Plant source					Isolation area	Isolating medium	Inhibiting ability against *C. manginecans* (%)	
		Species	Age	Root	Phyllode	Stem			Contact	Non-contact
EB-169	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	78.99	19.67
EB-75	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	83.54	19.61
EB-119	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	49.76	19.29
EB-65	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	65.79	19.28
EB-227	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	85.53	18.89
EB-71	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	52.26	18.75
EB-193	2/05/2015	*Acacia mangium*	4 years	v			Baserah compartement J015	LB Medium	76.17	18.69
EB-265	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	NA Medium	85.15	18.31
EB-182	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	38.97	18.18
EB-131	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	75.67	18.06
EB-222	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	LB Medium	88.24	17.78
EB-76	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	76.54	17.65
EB-121	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	60.87	17.53
EB-266	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	84.65	17.53
EB-2	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	NA Medium	72.60	17.31
EB-224	29/05/2015	*Acacia mangium*	2 years			v	Baserah compartement I027	LB Medium	71.37	17.18
EB-249	29/05/2015	*Acacia mangium*	4 Years			v	Baserah compartement I048	NA Medium	72.41	17.09
EB-269	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	NA Medium	82.18	16.44
EB-276	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	85.15	16.22
EB-114	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	77.78	16.07
EB-259	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	85.52	16.00
EB-61	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	81.48	15.91
EB-118	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	80.19	15.38
EB-127	26/3/2015	*Acacia mangium*	8 Months		v		Pelalawan TPK1	NA Medium	49.43	15.27
EB-192	2/05/2015	*Acacia mangium*	4 years	v			Baserah compartement J015	LB Medium	79.44	15.24
EB-261	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	89.66	14.55
EB-85	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	87.06	13.95
EB-36	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	24.27	13.81
EB-217	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	80.78	13.79
EB-46	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	75.54	13.58
EB-257	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	65.86	12.84
EB-58	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	85.00	12.84
EB-117	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	58.45	12.80
EB-50	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	55.79	12.60
EB-147	25/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	NA Medium	67.74	11.83
EB-35	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	82.01	11.72
EB-230	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	46.38	11.63
EB-100	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	68.82	11.54
EB-155	25/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	NA Medium	47.90	11.54
EB-189	2/05/2015	*Acacia mangium*	4 years	v			Baserah compartement J015	NA Medium	76.17	11.54
EB-191	2/05/2015	*Acacia mangium*	4 years	v			Baserah compartement J015	NA Medium	81.31	11.32
EB-15	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	78.86	10.93
EB-260	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	86.55	10.75
EB-251	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	LB Medium	72.07	10.71
EB-252	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	LB Medium	59.66	10.36
EB-88	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	88.81	10.26
EB-72	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	82.72	10.19
EB-55	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	83.13	10.00
EB-4	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C005	NA Medium	68.57	8.96
EB-37	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	63.18	8.77
EB-52	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	64.81	8.77
EB-103	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	21.89	8.58
EB-98	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	50.95	8.33
EB-221	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	LB Medium	88.24	8.33
EB-97	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	75.29	8.30
EB-162	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	87.39	7.89
EB-210	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	2.7	7.55
EB-232	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	83.40	7.41
EB-20	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	30.57	7.33
EB-267	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	84.65	6.98
EB-240	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	NA Medium	82.98	6.79
EB-23	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	LB Medium	21.40	6.53
EB-124	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	88.21	6.25
EB-199	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.97	5.71
EB-203	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.97	5.71
EB-107	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	18.87	5.59
EB-179	2/05/2015	*Acacia mangium*	4 years			v	Baserah compartement J015	NA Medium	70.77	5.56

Appendix 1 (continued)

EB Code	Isolating Date	Plant source					Isolation area	Isolating medium	Inhibiting ability against *C. manginecans* (%)	
		Species	Age	Root	Phyllode	Stem			Contact	Non-contact
EB-83	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	85.31	5.47
EB-47	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	83.30	5.36
EB-89	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	85.66	5.33
EB-206	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	44.39	5.33
EB-209	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	17.65	4.98
EB-92	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	82.73	4.88
EB-96	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	54.40	4.51
EB-44	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	26.78	4.36
EB-8	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	77.14	4.11
EB-228	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	82.55	3.77
EB-11	25/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartment C004	NA Medium	5.71	3.07
EB-154	25/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	NA Medium	41.60	2.93
EB-19	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	NA Medium	29.69	2.78
EB-21	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	LB Medium	9.61	2.74
EB-101	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	31.18	2.60
EB-246	29/05/2015	*Acacia mangium*	4 Years		v		Baserah compartement I048	LB Medium	44.14	2.56
EB-173	30/4/2015	*Acacia mangium*	3 years			v	Baserah compartement J029	LB Medium	70.30	2.53
EB-87	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	84.62	2.52
EB-63	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	81.20	2.45
EB-231	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	85.11	2.22
EB-43	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	64.44	2.22
EB-102	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	40.40	2.20
EB-229	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	NA Medium	84.26	2.18
EB-262	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	89.66	2.08
EB-247	29/05/2015	*Acacia mangium*	4 Years			v	Baserah compartement I048	NA Medium	28.28	1.84
EB-132	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	62.50	1.82
EB-105	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	6.79	1.73
EB-86	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	84.62	1.72
EB-106	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	LB Medium	15.09	1.52
EB-122	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	LB Medium	55.56	1.49
EB-94	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	-4.15	1.45
EB-129	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	77.57	1.45
EB-135	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	45.16	1.33
EB-195	2/05/2015	*Acacia mangium*	4 years		v		Baserah compartement J015	NA Medium	78.04	1.23
EB-248	29/05/2015	*Acacia mangium*	4 Years			v	Baserah compartement I048	NA Medium	45.52	1.15
EB-80	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	83.90	0.40
EB-14	25/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartment C004	NA Medium	82.86	0.00
EB-30	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	86.03	0.00
EB-32	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	83.70	0.00
EB-54	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	85.34	0.00
EB-82	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	85.66	0.00
EB-90	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	86.01	0.00
EB-99	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	32.70	0.00
EB-112	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	80.19	0.00
EB-113	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	84.54	0.00
EB-125	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	NA Medium	87.83	0.00
EB-130	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	81.37	0.00
EB-160	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	87.39	0.00
EB-205	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	81.31	0.00
EB-218	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	85.88	0.00
EB-268	3/06/2015	*Acacia mangium*	5 Years			v	Baserah compartement G023	LB Medium	85.15	0.00
EB-91	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	NA Medium	86.01	-0.87
EB-69	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	LB Medium	83.54	-0.88
EB-136	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	34.22	-0.96
EB-25	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	2.18	-1.33
EB-81	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	86.36	-1.43
EB-95	24/3/2015	*Acacia mangium*	10 Months		v		Mandau Compartement C009	NA Medium	29.66	-1.55
EB-59	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	84.96	-1.74
EB-215	29/05/2015	*Acacia mangium*	2 years		v		Baserah compartement I027	NA Medium	3.14	-2.31
EB-49	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	69.53	-2.56
EB-264	3/06/2015	*Acacia mangium*	5 Years	v			Baserah compartement G023	LB Medium	85.86	-2.63
EB-22	25/3/2015	*Acacia mangium*	1 Year		v		Teso East Compartment C027	LB Medium	16.72	-2.65
EB-200	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.97	-2.70
EB-126	26/3/2015	*Acacia mangium*	8 Months	v			Pelalawan TPK1	NA Medium	87.83	-2.74
EB-57	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	80.69	-2.83
EB-175	30/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	LB Medium	81.03	-2.86
EB-208	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	85.05	-2.86

Appendix 1 (continued)

EB Code	Isolating Date	Plant source					Isolation area	Isolating medium	Inhibiting ability against *C. manginecans* (%)	
		Species	Age	Root	Phyllode	Stem			Contact	Non-contact
EB-207	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	LB Medium	83.64	-3.03
EB-167	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	LB Medium	87.39	-3.12
EB-204	29/05/2015	*Acacia mangium*	2 years	v			Baserah compartement I027	NA Medium	78.04	-3.33
EB-165	30/4/2015	*Acacia mangium*	3 years	v			Baserah compartement J029	NA Medium	87.39	-3.33
EB-39	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	64.02	-4.07
EB-62	24/3/2015	*Acacia mangium*	10 Months	v			Mandau Compartement C009	NA Medium	82.72	-4.35
EB-38	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	LB Medium	64.85	-4.37
EB-234	29/05/2015	*Acacia mangium*	4 Years	v			Baserah compartement I048	LB Medium	82.55	-4.48
EB-190	2/05/2015	*Acacia mangium*	4 years	v			Baserah compartement J015	NA Medium	31.3	-4.49
EB-79	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	87.06	-5.02
EB-128	26/3/2015	*Acacia mangium*	8 Months			v	Pelalawan TPK1	NA Medium	54.75	-5.26
EB-24	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	18.78	-5.33
EB-78	24/3/2015	*Acacia mangium*	1 Year			v	Teso East Compartment C027	LB Medium	50.62	-5.91
EB-177	30/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	LB Medium	79.49	-6.35
EB-28	25/3/2015	*Acacia mangium*	1 Year	v			Teso East Compartment C027	NA Medium	86.90	-12.90
EB-178	30/4/2015	*Acacia mangium*	3 years		v		Baserah compartement J029	LB Medium	77.44	-13.24
			Total	143	59	76				